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THE RED CELL ANTIGENS OF THE HORSE

II. Antigens Defined by Immune Isoantibodies

By

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INTRODUCTION

In contrast to the large number of investigations on naturally occurring horse isoantibodies (reviewed by Adams, 1958) few have dealt with antibodies produced either during isoimmunisation of pregnancy or by experimental injection of red cells.

In their paper on haemolytic disease of newborn foals Coombs, Crowhurst, Day, Heard, Hinde, Hoogstraten and Parry (1948) showed that sera from mares which had given birth to foals with haemolytic disease showed a complex pattern of reactions, some sera agglutinating the red cells of a few horses, whilst one serum agglutinated the cells of all other horses with which it was tested. This suggested that several red cell antigens were implicated in this disease. Previously Lehnert (1939) had prepared high titre antibodies by injecting horses with red cells, and described the existence of several antigens, which he considered to form two main groups. In group A there was a principal receptor A_1, and five partial receptors A_2, A_3, A_4, A_5, and A_6, and a similar system existed with three partial receptors for group B. All the reagents for partial receptors were obtained by absorption and it may be that the reactions he observed were due to incomplete removal of antibodies by weakly reacting cells, so that the sera obtained still reacted with strongly reacting cells possessing the same antigen as the absorbing cell. However, it is impossible to decide this from the details given. Besides these agglutinins, Lehnert described three hemolysins and one other agglutinin but the relation of these antibodies to the anti-A and anti-B complexes is not clear. Balakrishnan (1953) also produced an immune serum by injection of red cells, but did not attempt to discover how many antibodies the serum contained. Podliachouk (1957, 1958), using sera obtained mainly from mares which had given birth to mule foals affected with haemolytic disease, has shown the existence of eleven antigens (many present in both the ass and horse) and investigated the antigenic relationship of these two species.

The present report describes the analysis of immune sera and the serological characteristics of the antigens and antibodies identified.

MATERIALS AND METHODS

Animals

The horses from which samples of blood were obtained included sixty-three of a herd of Exmoor, Shetland, Welsh, and cross-bred ponies, four Thoroughbred mares and a Thoroughbred colt.

Collection of blood samples

Cells and sera were collected in the manner described by Adams

(1958) except that acid citrate dextrose was always used as an anti-coagulant.

Sources of serum

Iso-immunisation of pregnancy. Serum was obtained as soon after foaling as possible, as it was found that the titre of anti-erythrocytic antibody in serum frequently falls rapidly once the foal has been born and has suckled. Blood samples were obtained from mares which had produced foals suffering from haemolytic disease, and from mares which were becoming immunised during late pregnancy. Thus in no case was serum obtained before the end of the first pregnancy in which iso-immunisation occurred.

Experimental iso-immunisation. Cells for injection were drawn with the usual aseptic precautions into anticoagulant, centrifuged, the supernatant plasma removed and the cells washed once with normal saline. Every effort was made to maintain sterility. The cells were then mixed with an equal volume of sterile normal saline, and injected into the external jugular vein, taking about ten minutes to give 300 ml of a 50 per cent suspension. The first injection was usually 500 ml of cell suspension, and subsequent injections of 300 ml were made at intervals of seven days until the titre of antibody had reached a satisfactory level. Four injections were usually sufficient. Occasionally a mild reaction characterised by shivering, sweating (particularly of the axillary skin) and increased restlessness was observed, in which event the transfusion was immediately stopped. The titre of antibody continued to rise until about four weeks after the last injection, and then started to fall slowly. In four animals it has been seen to fall to half the original titre in about five months.

Storage of serum. Some of the sera were obtained in 1947 or 1948, and were then freeze dried. However, sera stored at minus 20°C. since 1947 appeared to be still active, and subsequent samples of sera were stored in this way without preservatives.

Serological tests

Direct agglutination test in a saline medium. Undiluted horse serum causes washed horse red cells to form rouleaux and clump together, a property which disappears if the serum is diluted more than 1:4. These rouleaux may sometimes be broken up by diluting the mixture of serum and cells with saline on a glass slide and gently rocking, but it is often difficult to decide if the clumping is due to rouleaux formation, which may become very irregular and simulate agglutination closely.

Solutions of lecithin, which produce spherical transformations of red cells, were used in the hope that spherical red cells would not form rouleaux, but would still agglutinate. However, this method has not given reliable results. Some cells have been so altered that they were not agglutinated by agglutinins acting in a saline medium, even though they could be shown to be sensitised by the direct sensitisation test. Some cells are very refractory to the sphering action of lecithin, and all cells are refractory on the day they are bled, or the following day. Increase in the concentration of the lecithin solution causes lysis of the red cells. (Lattes, 1932 Ponder, 1948).

Many of the agglutinating antibodies formed in iso-immunisation of pregnancy or after experimental injection of red cells have a much

higher titre than 1:4, and in practice it has been possible to overcome the difficulties caused by rouleaux formation simply by diluting the sera. Saline agglutination tests were carried out by mixing one drop of horse serum, suitably diluted, with one drop of a 2 per cent suspension of washed red cells in a small tube (5 x 50 mm) and incubating at 37°C. for one hour. The sedimented cells were examined microscopically.

Agglutination tests were carried out using bovine albumin media (Diamond and Denton, 1945) and also trypsin-treated cells (Morton and Pickles, 1951). Reading the albumin test was difficult because of the formation of rouleaux, although this was not so marked as in undiluted horse serum. A 20 per cent solution of bovine albumin gave the best results.

Indirect sensitisation test. This was performed as described by Adams (1958), except that rabbit anti-horse globulin sera were always used.

Haemolytic test. This test was performed by the method described by Chaplin, Wallace and Chang (1956). Fresh horse serum was usually used as a source of complement, but guinea-pig or rabbit serum could be used with little difference to the result.

Absorptions. Absorptions were done by the method described by Adams (1958) except that the process of absorption was always repeated four times at 37°C, unless described otherwise in the text.

Direct conglutination test. (Hole and Coombs, 1947). Conglutination of horse red cells can be caused by mixing 0.1 ml of a 1 per cent suspension of washed cells in a 2 ml tube with 0.1 ml of heated antiserum, 0.1 ml of fresh unheated horse serum usually diluted to 1:4 to give four minimal conglutinating doses, and 0.1 ml of heated bovine serum, diluted to 1:10, which had been previously absorbed with washed horse red cells. The tube was placed in a 37°C water bath for forty-five minutes, shaken frequently, and then centrifuged at 1,500 r.p.m. for 20 seconds. The reaction was read by tapping the tube gently and causing the contents to rise in the supernatant fluid either as clumps (a positive reaction) or with no observable macroscopic clumping (negative reaction). Various grades of conglutination can be observed.

Complement fixation tests. The fixation of complement by a horse red cell sensitised by isoantibody was shown by using both a haemolytic indicator system (sheep red cell and a rabbit immune antibody) for guinea pig complement or a conglutinating system (sheep red cells and inactivated bovine serum) for conglutinating complement. The conglutinating complement absorption test was performed by incubating a 1 per cent suspension of horse cells with heated antiserum at 37°C for thirty minutes and then centrifuging at 3,000 r.p.m. for one minute. The supernatant was then added to the indicator system composed of sheep cells and heated bovine serum and incubated in a water bath at 37°C for forty-five minutes, when the tubes were centrifuged for twenty seconds at 1,500 r.p.m. and read.

Absorptions. Absorptions were usually carried out by incubating equal volumes of packed cells, washed four times, with heated undiluted serum at 37°C in a water bath for ten minutes. After each absorption the tube was centrifuged at 3,000 rpm. for ten minutes. Repeating this process a further three times usually removed all the antibody with

TABLE 1

ANALYSIS OF SERUM FROM MARE (CASE 5): REACTIONS BETWEEN SERA AND TEST CELLS

Serum from mare (Case 5) absorbed with cells from:	*Cells*															*Antibodies present in serum*
	Ke.	*Bar.*	*Hes.*	*Hen.*	*St.*	*S.A.*	*Bet.*	*An.*	*Bel.*	*Bre.*	*Pr.*	*Mo.*	*Hi.*	*Gr.*	*Spir.*	
Keith	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	anti-2
Barbara	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	anti-2
Hester	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	anti-2
Henry	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	anti-2
Stella	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	anti-2
Steady Aim	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	anti-2
Betsy	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	anti-2
Anthea	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	anti-2
Belinda	+	+	+	+	+	+	+	+	—	—	+	+	+	+	+	anti-1 plus anti-2
Brenda	+	+	+	+	+	+	+	+	—	—	+	+	+	+	+	anti-1 plus anti-2
Princess	+	+	+	+	+	+	+	+	—	—	—	+	+	+	+	anti-1
Morgan	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	none
Hilda	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	none
Greta	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	none
Spirella	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	none
Antigens present on cells.	1	1	1	1	1	1	1	1	None	None	2	1+2	1+2	1+2	1+2	

which that particular cell was capable of reacting. Any deviations from this method are noted in the text.

RESULTS

Sera from four mares which had given birth to foals subsequently affected with haemolytic disease of the newborn and one serum from a gelding which had been injected with horse red cells were analysed. Seven antibodies of different specificities have been obtained. Other sera, not described here, have been found to contain some of these seven antibodies and there are further antibodies whose characteristics have not yet been determined. The haematological and clinical features of haemolytic disease of the newborn foals of mares 2, 5 and 6 were described by Coombs *et al.* (1948).

Analysis of Serum from Mare 5

The serum in amounts of 1 ml was absorbed with cells from each of fifteen different horses and the absorbed sera, diluted to 1:10, were tested for agglutination of every one of the fifteen cells by saline agglutination and indirect sensitisation tests. The pattern of reactions by the indirect sensitisation test is shown in Table 1 and may be explained on the basis of two antigens, identified as 1 and 2. As will be shown later (Table 2) these purified antibodies did not contain any saline aggutinin, although, as Coombs *et al.* (1948) had previously demonstrated, there is a saline agglutinin in the serum of mare 5. It has not been possible to separate this agglutinin so far by absorption methods.

The purified antisera were then tested against cells from a further fifty-three horses. Absorption with each of those cells which reacted (forty-one) removed all antibody from the serum, thus providing some assurance of purity. Analysis of serum from two other mares has shown the presence of anti-1 together with anti-2. Anti-1 has been found together with other antibodies in the serum of five mares, and anti-2 in the serum of four. Eighteen sera have been analysed.

Analysis of Serum from Mare 2

This serum was absorbed with cells from each of eighteen horses and an investigation similar to that of the serum of mare 5 was carried out. Three antibodies, anti-3, anti-4 and anti-5, were obtained from this serum, and others are present which cannot be isolated with the horse cells available. Absorption of purified antisera with a positive cell removed all antibody in each case. Neither anti-3 nor anti-4 have been found in any of the other sera analysed, whereas anti-5 has been found in four other sera.

TABLE 2

SEROLOGICAL PROPERTIES OF ANTIGEN/ANTIBODY SYSTEM

Antibody and source	*Type of test cell*	*Titre of pure antiserum measured by:*				
		Saline test	*I.S.T.*	*Albumin test*	*Conglutination*	*Lytic test*
Anti-1 Case 5	Conglutinable	—	16	8	8	2
	Inconglutinable	—	16	8	—	—
Anti-2 Case 5	Conglutinable	—	64	32	32	8
	Inconglutinable	—	64	32	—	—
Anti-3 Case 2	A	16	64	32	B	—
Anti-4 Case 2	A	—	8	2	—	—
Anti-5 Case 2	Conglutinable	—	512	64	128	32
	Inconglutinable	—	512	128	—	—
Anti-6 Mare 5	A titrated against the same cell	—	2,000	512	512	32
Anti-6 Gelding Ar		16,000	32,000	1,000	B	64
Anti-7 Case 6	A	—	8	2	—	—

A. All cells with these antigens behave in the same way.
B. The titre of these sera by the conglutination test is no higher than that by the saliva agglutination test.
I.S.T.=indirect sensitisation test.

Analysis of Serum from Mare Pb

Serum from this mare agglutinated cells from about 70 per cent of all the horses tested, and absorption with cells from each of twenty horses whose cells reacted removed all agglutinin. It has not been possible to fractionate this serum further, and it is likely that it contains only one isoantibody, identified as anti-6. This antibody has been found, alone or with other antibodies in the serum of four other mares, and has been produced by injection of red cells from a 6-positive mare into a 6-negative gelding.

Analysis of Serum from Mare 6

Serum from this mare contained several antibodies, most of them active only at high concentrations, and which were not therefore suitable for use as test-reagents because of the complications of rouleaux formation. One antibody, however, had a titre of 1:8 by the indirect sensitisation test and defined a low frequency antigen. Absorption with each of all the available horse cells (eight) which possessed this antigen removed all antibody, which was called anti-7, and which has not been found in any other of the eighteen sera analysed.

Serological Characteristics of the Antibodies Isolated

Agglutinating antibodies to antigens 3 and 6 have been found and sera containing anti-6 have been isolated which do not agglutinate red cells in a saline medium, but can be shown by means of an anti-globulin serum to sensitise cells. One anti-6 serum, from a mare Pb, exhibited a prozone, failing to cause agglutination at high concentrations. Anti-6 from mare 5 (Table 2) which is incapable of causing agglutination in a saline medium, will inhibit the action of the saline agglutinin present in the serum of a gelding ("Arthur"). Although several examples of anti-1, anti-2 and anti-5 have been found, these have all had the same properties as those of the first examples of these antibodies, whose derivation has been described above. These properties are set out in Table 2.

The albumin test, although difficult to use because of rouleaux formation, tends to give comparable titres to those obtained by the indirect sensitisation test. Trypsin, on the other hand, fails to make red cells agglutinable by antibodies which do not cause agglutination in a saline medium. The length of incubation with trypsin was increased but no specific agglutination was produced. After incubation for one hour all cells were agglutinated by all sera.

The conglutination test on those cells with which it acts also gives titres comparable to those obtained by the indirect sentisation test; lytic tests give titres which are usually four tubes lower. However, some cells known to possess the antigen were not conglutinated.

Behaviour of Cells in the Conglutination Test

As mentioned above, sera containing antibodies to antigens 1, 2, 5 and 6 may cause conglutination or lysis of cells from horses with the appropriate antigens, and sensitised cells may be shown to fix complement. Cells from some horses, however, which can be shown to react with an anti-serum by the indirect sensitisation test, do not give the reactions of conglutination or lysis. This could be due to the presence of two antibodies in the reagent, one reacting by the indirect sensitisation test alone with one class of cells, the "inconglutinable" cells, and the other reacting with all other cells by conglutination, lysis, and the indirect sensitisation test. In fact this was not so; the sera were shown to contain only one antibody, since absorption with the inconglutinable cell removed the antibody causing hemolysis or conglutination of the other cells. Alternatively it is possible that there is some characteristic difference between the two types of cell which prevents the reaction of the inconglutinable or allows the reaction of the conglutinable cell. Furthermore, it can be shown that sensitised inconglutinable cells do not fix complement as much as do sensitised conglutinable cells, and that the former cells absorb less antibody.

TABLE 3

DIFFERENCES IN REACTION OF CELLS FROM VARIOUS HORSES TO THE SAME ANTIBODY

Antibody	*Reaction*	*Horse cell*							
		San	*Box*	*Alex*	*S.A.*	*Bun*	*Bar*	*Spir*	*Hil*
anti-1	*I.S.T.*	+	+	+	+	+	+	+	+
	Conglutination	+	−	+	−	−	−	−	+
	Lysis	+	−	+	−	−	−	−	+
	Complement fixation	+	−	+	−	−	−	−	+
anti-2	I.S.T.	−	+	+	−	−	−	+	+
	Conglutination	−	+	−	−	−	−	+	+
	Lysis	−	+	−	−	−	−	+	+
	Complement fixation	−	+	−	−	−	−	+	+

A consequence of this phenomenon is illustrated in Table 3, which shows some of the possible reactions of horse cells with isoantibodies. A cell which is inconglutinable for one antigen system is not necessarily inconglutinable for another, although it may be. In a series of sixty eight horses all possible combinations

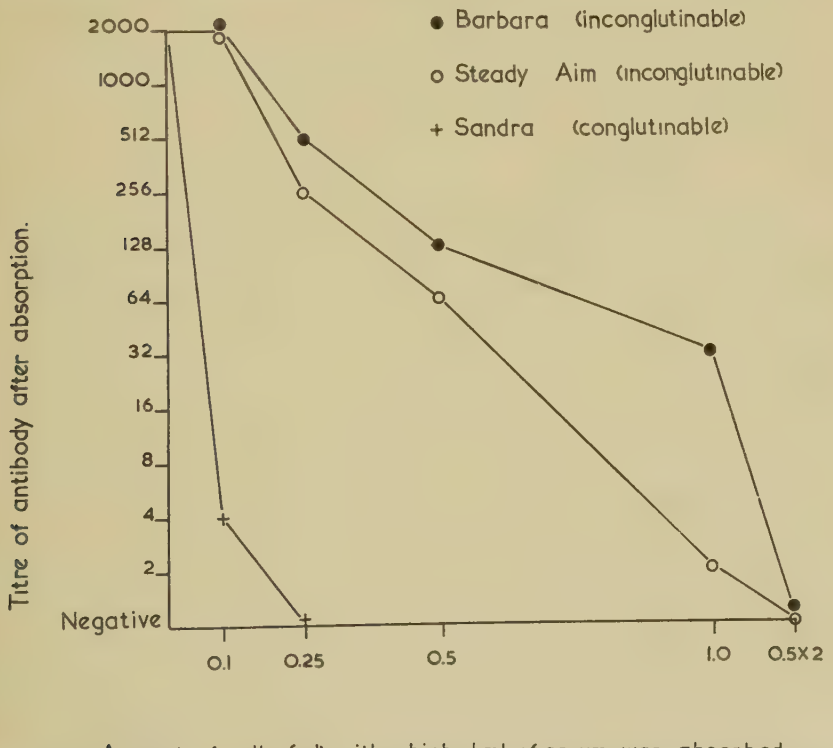
of reactions were found. This phenomenon is related to a particular antigen, and is not a general property of the cell. No horse has been found whose cells react with an antiserum by conglutination and hemolysis but not in the indirect sensitisation test.

Investigation of the Mechanism of the "Inconglutinable" Cell Phenomenon

Absorption experiments. Absorption of iso-antiserum with either inconglutinable or conglutinable cells, and then testing the absorbed sera with both types of cell will indicate if the serum is a mixture of antibodies. By absorption of fixed amounts of antiserum with increasing amounts of horse cells, a rough comparision may be made between two cells of the relative amounts of antigen available for fixation of antibody (Boursnell, Heard and Rizk, 1955; Heard, 1955; Joysey, 1955).

One ml amounts of the antiserum were absorbed with 0·1 ml, 0·25 ml, 0·5 ml and 1·0 ml. amounts of packed cells which had been washed in saline four times. The absorbed sera were then titrated, using the indirect sensitisation test or the conglutination test. It

Fig. 1.



Absorption of anti-1 by conglutinable cells (Sandra) and inconglutinable cells (Steady Aim and Barbara).

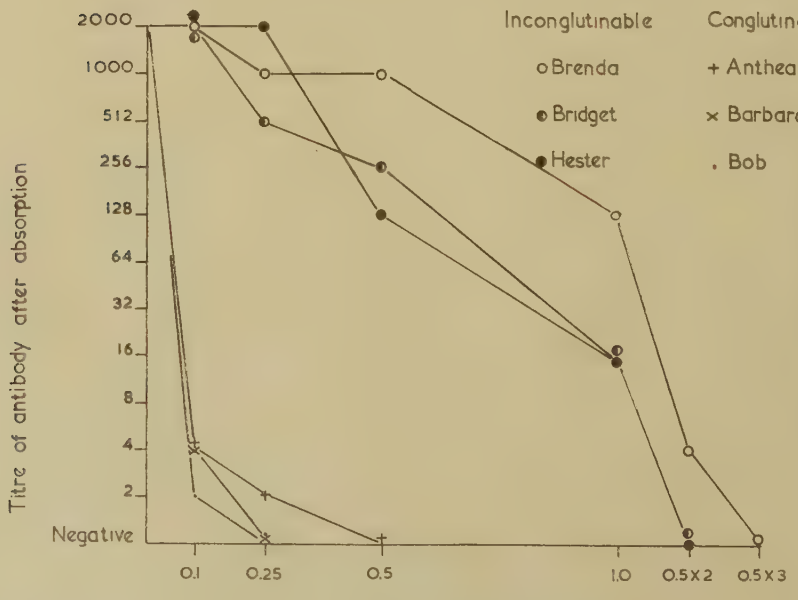
was often necessary, particularly with inconglutinable cells, to complete the absorption by absorbing 1 ml of serum twice or three times with 0·5 ml. of washed, packed cells.

As shown in Figure 1, absorption with inconglutinable cells finally removed all the antibody reacting with the conglutinable cell as detected by the indirect sensitisation test, and also by conglutination, or lytic tests, not shown in this figure. In contrast to the results obtained by Heard (1955) the titre of the absorbed serum was the same when tested with conglutinable as with inconglutinable cells, except towards the end of the absorption when very little antibody remained and the conglutinable cell seemed more likely to give a positive reaction than the inconglutinable cell. Heard, however, was using saline agglutination tests, which are less sensitive than the indirect sensitisation test in indicating combination of antigen with antibody.

Comparable absorption tests have been performed with antigen systems 1, 2, 5 (Fig. 2) and 6 (Fig. 3). Antigens 1, 2 and 5 show the inconglutinable cell phenomenon, but no cell has been found which will not be conglutinated or lysed by anti-6, but can be shown to possess the antigen by the indirect sensitisation test. All 6 positive cells absorb anti-6 to approximately the same extent (Fig. 3).

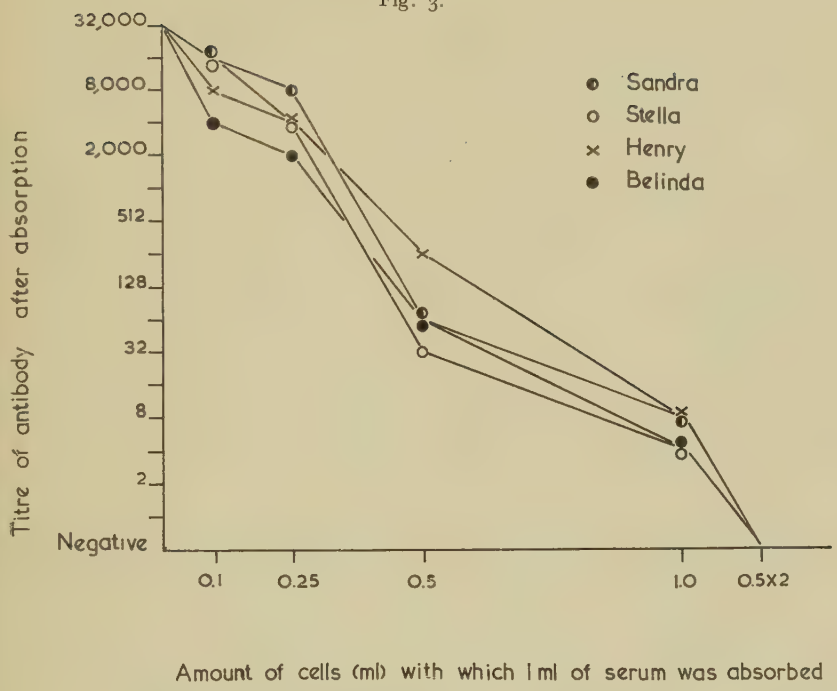
Some appreciation of the crudity of absorption experiments as

Fig. 2.



Absorption of anti-5 with conglutinable and inconglutinable cells.

Fig. 3.



Absorption of Saline agglutinating anti-6 with various cells.

a measure of the amount of antigen capable of reacting may be obtained by observing the amount of scatter in the titres after absorption with the same quantities of cells (Fig. 3). Absorption of 1 ml of saline agglutinating anti-6 with 0·25 ml of "Sandra's" cells reduced the titre as determined by the indirect sensitisation test by two dilutions from 1:32,000 to 1:8,000, whereas absorption of the same amount of serum with the same amount of "Belinda's" cells reduced the titre by four dilutions. This cannot be a reflection of a difference in the absorbing ability of the two cells, since absorption of 1 ml. of serum with 0·5 ml of each of these cells reduced the titre by the same amount to 1:64. A difference of two doubling dilutions was usual, and of four doubling dilutions not uncommon. However, this may be contrasted with the constant finding that absorption of an iso-antiserum with 0·1 ml of a conglutinable cell removes almost all of the antibody, reducing its titre from 1:2,000 to 1:4 whereas absorption with 0·1 ml. of an inconglutinable cell does not remove a detectable amount of antibody (Fig. 2).

It has been noted that it is usually necessary to complete absorption with inconglutinable cells by absorbing twice or three times with 0·5 ml of washed packed cells, which may be considered to be a more efficient way of doing the absorption. These absorption experiments unduly emphasize the difficulty of removing all antibody with

TABLE 4

TITRATION OF HORSE COMPLEMENT AFTER INCUBATION WITH SENSITISED CELLS

To show differences in the ability of conglutinable and inconglutinable cells to fix complement

Complement absorbed by horse cells:		*Number of absorptions*	*Reciprocal of dilutions of incubated horse complement*									
			1	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*Saline control*
Bob	conglutinable cells	1	4	4	4	4	2	0	0	0	0	0
		2	2	0	0	0	0	0	0	0	0	0
Bunty		1	4	4	4	4	0	0	0	0	0	0
		2	0	0	0	0	0	0	0	0	0	0
Norma	inconglutinable cells	1	4	4	4	4	4	4	3	3	0	0
		2	4	4	4	4	4	4	3	0	0	0
Phillipa		1	4	4	4	4	4	4	3	0	0	0
		2	4	4	4	4	4	3	0	0	0	0
Princess		1	4	4	4	4	4	4	3	0	0	0
		2	4	4	4	4	4	3	2	0	0	0
Unabsorbed		–	4	4	4	4	4	4	4	3	0	0

Degrees of conglutination.

4 One large clump.
3 Many macroscopic clumps, no free cells.
2 Many macroscopic clumps, free cells.
0 No macroscopic clumps.

larger amounts of cells compared to repeated absorption with smaller amounts, and it would be unwise to assume that the relative amounts of antigen on the two types of cells are proportional to differences between the amounts of the cells needed to remove all antibody from a certain amount of serum.

Complement fixation by conglutinable and inconglutinable cells. Sensitised conglutinable cells were found to fix more of the complement present in horse serum than did sensitised inconglutinable cells. Incubation with 0·1 ml of a 2 per cent suspension of conglutinable cells sensitised with undiluted antiserum would reduce the titre of complement, as measured with a sheep cell and bovine serum indicator system, from 1:128 to 1:16 (Table 4) and a second absorption with the same amount of sensitised cells would remove all the complement. Incubation with the same amount of sensitised inconglutinable cells however, reduced the titre of complement from 1:128 to 1:64 at the most and sometimes did not alter the titre at all.

CONCLUSIONS

Seven red cell antigens have been identified by means of antisera from immunised horses, and these have been shown to be independent of the antigens defined by naturally occuring antibodies. It has not so far been possible to place the antigens in allelic series.

It is possible for two horses, both of which possess the same antigen, to differ in the amount of that antigen which is available for reaction with antibody, as demonstrated by the failure of conglutination or lysis to occur with the cells of one of the horses.

The phenomenon of inconglutinability is a property of the antigen and not of the cell itself, and may explain Lehnert's failure to relate antibodies causing lysis to those causing agglutination.

It has been shown that sensitised inconglutinable cells do not fix complement as fully as do sensitised conglutinable cells, and that inconglutinable cells absorb antibody much less readily. A possible explanation for the failure of the conglutination test is that a certain minimal amount of fixed complement is necessary to produce conglutination or lysis, and that there is insufficient antigen available on the inconglutinable cell to fix this minimal amount of complement, even when the cell is fully sensitised with antibody.

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CLINICAL BIOCHEMISTRY OF FARM ANIMALS

I. Proteins and Amino-acids of the Body Fluids of Cattle and Sheep

By

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INTRODUCTION

Blood and urine, by virtue of their accessibility, are by far the most common body fluids used for clinical and research purposes. There appear to be very few published reports, however, on the free amino-acids occurring in blood plasma and urine of cattle and sheep, either in health or disease (Holdsworth, 1956).

Doak (1952) analysed herbivore urine for the main groups of nitrogenous constituents and found that the amino-acid fraction accounted for 10·5 to 16 per cent of the total nitrogen, being second only to urea nitrogen. Bathhurst (1952) determined seventeen amino-acids by microbiological assay and found that over 90 per cent of the total amino-acid nitrogen was due to glycine. The results of Bathurst (1952) differ from those obtained on human urine by Dunn, Camien, Shankman and Black (1947). Cousins (1950) reported that paper chromatograms of wether urine showed that glycine was virtually the only amino-acid present, although faint spots were seen occasionally, indicating that traces of other amino-acids were to be found from time to time. Walker (1952) found only glycine in desalted adult bovine urine, but in milk-fed calves other spots appeared on paper chromatograms, and these ninhydrin positive materials corresponded to glutamic acid, taurine, alanine, glutamine and sometimes aspartic acid.

The results that Walker (1952) obtained for the free amino-acids of bovine and sheep plasma were similar to those obtained by Gordon (1949) for bovine serum, and even to those present in human plasma (Dent and Walshe, 1954). There appears to be no literature on the variation in free amino-acid content of bovine and sheep plasma as a result of disease.

Although not many accounts have been published on the protein fractionation of sheep and cattle serum by paper electrophoresis, some attention has been paid to the changes in the protein electrophoretic patterns brought about by disease, and starch gel electrophoresis has been carried out on normal serum (Ashton, 1957, 1958). Column electrophoresis has been used with certain animal sera (Mould and Stephen, 1958).

Paper electrophoresis has shown that there are two γ-globulin fractions in cattle serum (Boguth, 1953a; Campbell, 1957; Rooney, 1957). This technique enabled the passage of colostrum γ-globulin through the intestinal wall into the blood to be demonstrated (Boguth, 1953b).

The present paper is an attempt to prepare the way for the use of amino-acid patterns on two dimensional paper chromatograms

as a further diagnostic aid in the diseases of cattle and sheep, and to extend the use of paper electrophoresis, as another diagnostic aid, by employing improved techniques. The importance of these two aids has been well established in the human field (Dent and Walshe, 1954; Smith, 1958; Antweiler, 1957). Work on certain pathological conditions (Davies, 1958) will be reported in a subsequent paper.

METHODS

Blood samples. These were obtained by jugular venepuncture. For plasma preparation from blood (10 ml.) the anticoagulant was 30 per cent potassium oxalate solution (0·2 ml.). Serum and plasma samples were stored in the deep freeze at −20°c.

Urine samples. These were collected in bottles containing a small quantity of chloroform. After collection the samples were covered with a layer of toluene and frozen.

Removal of protein. In the case of the plasma, 95 per cent alcohol (8 vols.) was added to 1 vol. and, after initial shaking, followed by standing for half an hour, the precipitates were filtered off. For urine, samples were made 80 per cent (by volume) by adding ethanol, allowed to stand, cleared by centrifuging, evaporated, and the 10 per cent *iso*-propanolic solution of the residue desalted.

Hydrolysis. Since difficulties were encountered with the direct chromatography of sheep and cattle urine, acid hydrolysis was carried out. Urine (5 ml.) was tested first for absence of protein by 20 per cent salicylsulphonic acid solution, made 6N with hydrochloric acid and heated overnight in a sealed tube at 105°C. After filtration and evaporation, repeated several times after taking up in water, the 10 per cent *iso*-propanolic solution was desalted. Protein-free plasma extract (5 ml.) was, on occasions, treated in the same way.

Desalting. The desalting apparatus* was used according to Smith, Stevens and Jepson (1956) and Smith (1958).

Chromatography. Whatman No. 4 paper ($18\frac{1}{4}'' \times 22\frac{1}{2}''$) was used for two dimensional chromatography. The procedure using phenol/water (80/20) in a 1 per cent ammonia atmosphere as the first solvent and *n*-butanol/acetic acid/water (62/12/26) as the second, and square cross-sectioned "Dural" troughs for descending chromatography was much the same as that reported elsewhere (Coulson, 1953, 1955). The second solvent system contained 0·1 per cent ninhydrin (w/v). The chromatograms, in their troughs, were transferred to a drying oven and dried after each run in a stream of warm air, just sufficient solvent being used for each run so that little remained in the troughs. The temperature of the chromatography room was 20° ± 1°.

The plasma extract (0·3 ml.) was obtained originally from 5 ml. of plasma. The urine extract (0·3 ml.) was equivalent to 0·75 ml. of the original urine, as was the hydrolysate of the urine.

One dimensional chromatography was used, where necessary, in conjunction with multiple dipping techniques (Smith, 1958).

Paper electrophoresis. A horizontal type electrophoresis tank† was used in which three strips, 5 cm. wide, could be placed. Carbon electrodes were used. A constant D.C. source was supplied by a power pack. The colour density curves were prepared by using a double beam reflecting automatic recording densitometer with integrator§. For the stabilising medium 'Oxoid' cellulose acetate strips (30 × 5 cm.) were used, (Kohn, 1957 a, b). They were not made transparent since this was not necessary with the reflectance method. A pH 8·6 barbiturate buffer was used (barbitone, 1·84 g./l; soluble barbitone, 10·31 g./l.) Glycerol (20 ml./1) was mixed with the buffer before use.

The following voltages gave adequate separation for quantitative analysis without distortion: 210 v. for 8 hrs. or 240 v. for 7 hrs. The current was 2·5 mA approximately (i.e. 0·5 mA per cm. width).

Fig. 1. Amino-acid pattern of cattle and sheep plasma. Two dimensional paper chromatogram.

1st. Direction: Phenol: Water/NH_3. 2nd. Direction: n-Butanol/Acetic Acid/Water. Location reagent: ninhydrin. Colour intensities given inside brackets (O-I: trace; I: very weak; II: weak; III: medium; IV: strong; V: very strong). 1. Glutamic acid (IV). 2. Serine (IV). 3. Glycine (IV). 4. Threonine (III). 5 Alanine (V). 6. Citrulline (III). 7. Glutamine (III). 8. Histidine (II). 9. Lysine (II). 10. Arginine (II). 11. Proline (II). 12. Tyrosine (II). 13. α-Amino-butyric acid (II). 14. Valine + methionine (V). 15. Phenylalanine (I). 16. Leucine (s) (V). 17. Aspartic acid (1). 18. Dopa (?) (o-I). 19. Asparagine (o-I). 20. Unknown (O-I). 21. Unknown (O-I).

Fig. 2.

Amino-acid pattern of hydrolysed cattle and sheep urine. See Fig. 1 for other information.

1. Cysteic acid (O-I). 2. Aspartic acid (II). 3. Glutamic acid (IV). 4. Dopa(?) (I). 5. Serine (III). 6. Glycine (V). 7. Threonine (II). 8. Alanine (III). 9. Hydroxyproline (II). 10 & 10a. Methionine sulphoxide and sulphone. (I). 11. Histidine (II). 12. Arginine (II). 13. Lysine (II). 14. Unknown (I) 15. β-alanine (I). 16. Proline (I). 17. Tyrosine (I). 18. γ-amino-n-butyric acid (II). 19. β-amino-*iso* butyric acid (I). 20. Valine + methionine (II). 21. Unknown (II). 22. Phenylalandine (I). 23. Leucine(s) (II). 24. Unknown (III).

The serum (15 μl.) was applied, using the edge of a thick microscope slide, as a band to the buffer saturated 'Oxoid' strip. The slide was left in contact with the strip for about one minute and during this time it was moved carefully to and fro so that a uniform band of about 0·5 cm. width was formed. Even distribution of the sample at the start is more difficult than when ordinary filter paper is used. Excess buffer was removed by blotting between filter paper and the electrophoresis strips, held in the form of paper cylinder by a paper clip, were dried at 90° for 5 mins. Dry strips were immersed in Azocarmine B solution (0·75 g. in methanol/glacial acetic acid/water: 50 ml./10 ml./40 ml; 10 mins.), washed with a methanol/acetic acid mixture (9/1, v/v; 5 mins.) followed by a water/acetic acid mixture (9/1, v/v; 5 mins.), and then dried between filter paper under weights. The method was similar to that of Plückthun and Götting (1951). The same batch of Azocarmine B was used throughout. Loeffler and Wunderly (1953) showed that, of the commoner dyes,

uptake of Azocarmine B by serum globulins comes the closest to that of the albumins.

* Messrs. Aimer Products, Ltd., London.

† Messrs. Shandon & Co. Ltd., London.

§ Messrs. Joyce, Loebl & Co., Newcastle-upon-Tyne: 1958 Model.

RESULTS

Amino Acids

Plasma. The plasma chromatograms of a large number of cattle and sheep showed that the qualitative patterns of amino acids were identical for the two species. A representative map is given in Fig. 1. Quite a number of plasma samples from lactating ewes were examined shortly after lambing and they all seemed to contain larger amounts of basic amino-acids (arginine, histidine, lysine). There appeared to be no change of the amino-acid pattern with age. Serum gave stronger amino-acid patterns.

Hydrolysis of the plasma extract resulted in an overall strengthening of the colour intensities (Fig. 2). Glutamine (No. 7) and asparagine (No. 19) as well as spots No. 20 and No. 21 disappeared (Fig. 1). Cyst(e)ine and methionine were confirmed by hydrogen peroxide oxidation (formation of cysteic acid and methionine sulphone respectively). The following amino-acids were confirmed by other reagents: proline (isatin), citrulline (Ehrlich: also giving urea), arginine (Sakaguchi), histidine and tyrosine (diazo) (Smith, 1958).

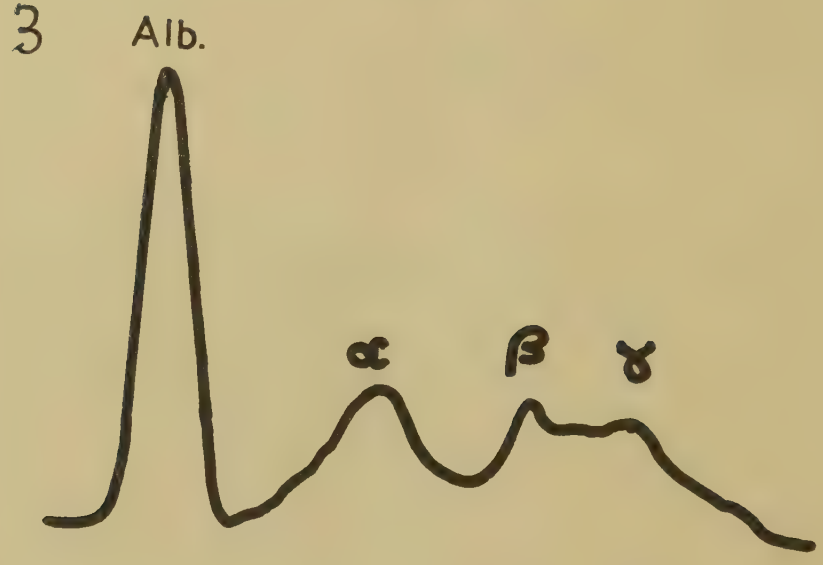
TABLE 1

COMPOSITION OF APPARENTLY NORMAL CALF SERUM (RELATIVE %)

Animal No.	*Albumin*	*Globulins*		
		α	β	γ
1	43	22	10	25
2	44	22	12	22
3	46	20	12	22

Urine. Chromatograms of cattle and sheep urine of up to 0·5 ml. of urine revealed the presence of little else than glycine. More urine increased the amount of ninhydrin-positive material detectable, but except in a few cases, there was considerable pattern disturbance resulting from interfering substances. In these it was possible to detect glutamic acid, threonine, alanine, leucine(s), phenylalanine and probably valine + methionine and β-amino-*iso*-butyric acid. The interfering substances were probably urinary ampholytes (such as conjugates) other than amino-acids. During desalting of the

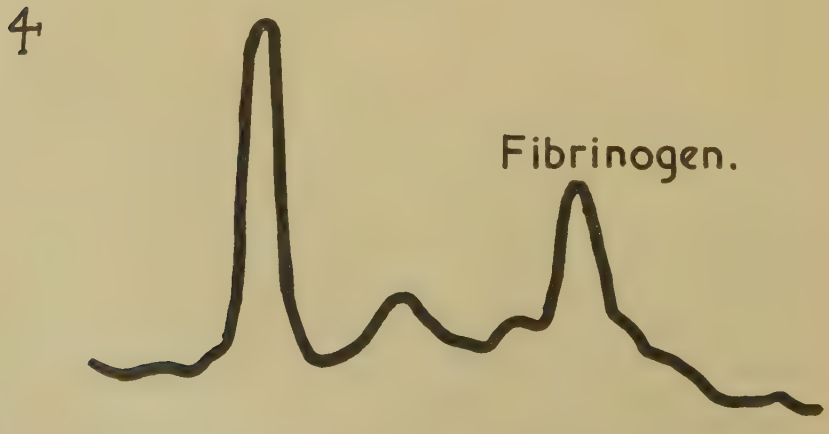
Fig. 3.



Colour density curve of an electrophoretogram of calf serum. Medium: "Oxoid" strip. Stain: Azocarmine B.

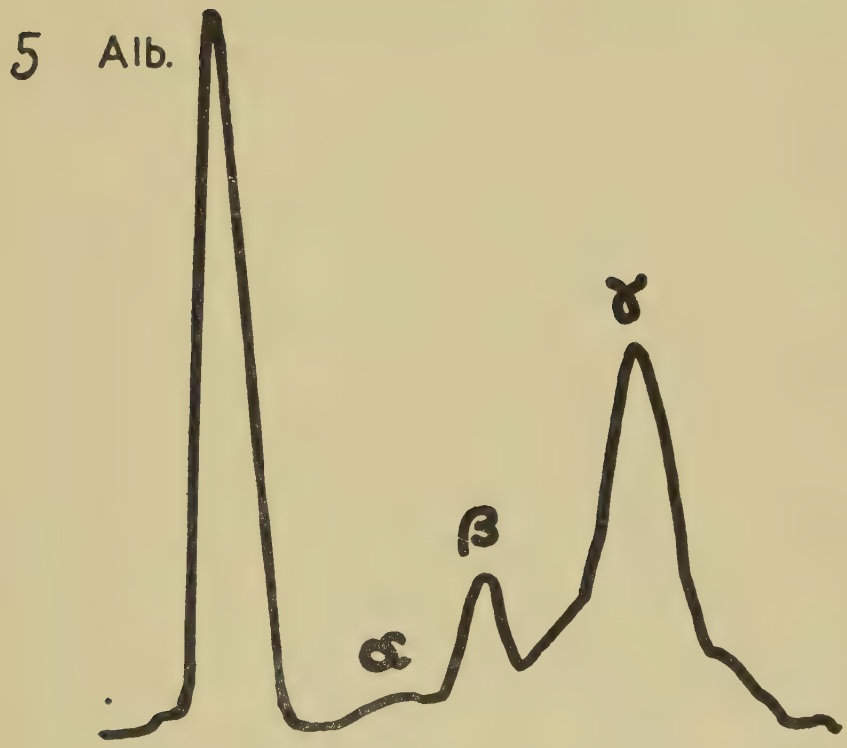
urine the current did not fall below 0·4 amp. whilst the plasma extract current fell to 0·1 amp. quite rapidly. The hydrolysis of the urine led to a great increase in the number of ninhydrin positive spots (Fig. 2). A further reagent was used to confirm hydroxyproline (Smith, 1958).

Fig. 4.



Colour density curve of an electrophoretogram of calf plasma.

Fig. 5.



Colour density curve of an electrophoretogram of sheep serum.

Proteins

The results for the electrophoresis of calf serum are given in Table 1 and those for five apparently normal sheep in Table 2. Typical curves obtained by the "scanning" of normal electrophoretograms of calf serum and plasma and sheep serum are given in Figs. 3, 4 and 5.

DISCUSSION

Results for plasma amino-acids agree reasonably well with those of Walker (1952) and with the serum results of Gordon (1949). There are, however, some differences. Both these authors detected taurine, but did not detect citrulline, asparagine or cyst(e)ine. Using the method given in this paper, taurine was not detected in any normal plasma examined, but citrulline was always detected. Asparagine was found in low concentration in the majority of plasmas examined. The presence of cyst(e)ine was only confirmed by one dimensional paper chromatograms in *n*-butanol/acetic acid/

water and by prior oxidation of the plasma extract before chromatography. Tryptophan was not detected by Walker (1952) or by us, although Gordon (1949) reported its presence in serum. Since our method of extraction was similar to that of Walker (1952), it is possible that tryptophan is lost during the extraction. The identity of the spot under proline (No. 21, Fig. 1), which is acid-labile, was not established.

Urinary amino-acid chromatograms have tended to confirm earlier findings as far as glycine is concerned. A high proportion of the amino-acids present in cattle and sheep urine is free glycine. However, it is clear from those chromatograms, which had fewer interfering substances present, that glutamic acid, serine, threonine, alanine, valine + methionine, phenylalanine, the leucine(s) and probably β-amino *iso* butyric acid were present, although in much smaller quantities. Previous authors seem to have failed to examine the urine very carefully. The large increase of ninhydrin-positive spots on the hydrolysis of sheep and cattle urine shows clearly that a substantial proportion of the amino-acids are present in a bound form. The increase in glycine shows that much of this amino-acid is in the bound form. Bathurst (1952) points out that hippuric acid will be responsible for quite a fair portion of the increase. Both glycine peptides and other glycine conjugates are probably the source of the remaining glycine increase. The other amino-acids present in the bound form are probably present in the form of peptides and conjugates (Bathurst, 1952; Williams, 1948). The ingestion by ruminants of fair quantities of benzenoid compounds which occur in the plant world (e.g. simple phenolic compounds, quinones, tropolones and simple polymers; flavonoids; tannins; lacs; alkaloids; lignins) may be responsible for the excretion of large amounts of glycine and other amino-acids as conjugates (Williams, 1948).

The results available for serum proteins of some apparently normal calves and sheep by the method reported in this paper do differ somewhat from those of other workers (Boguth, 1953a; Kutáček and Koloušek, 1953; Rooney, 1957; Campbell, 1957). It must be borne in mind that variation in conditions of separation and determination can result in variation of results. The conditions of separation, medium and method of measuring the protein-dye complex colour density did differ. These bovine and sheep serum protein separations on cellulose acetate membrane strips are apparently the first ones to be reported. The main divergence lies in the value of the α-globulin fraction. The values for the calf sera are consistently some 50 per cent higher than those for adult animals reported by Boguth (1953a), Campbell (1957) and Rooney (1957). This may be explained by the report of Pedersen (1945) that calf serum had a higher "fetuin" (a protein) content than did adult bovine serum. He found that "fetuin" migrated with the

α-globulin. The results with sheep serum show a higher γ-globulin than that reported in the literature. It should be noted that there is a high incidence of liver fluke infestation in the North Wales

TABLE 2

COMPOSITION OF APPARENTLY NORMAL SHEEP SERUM (RELATIVE %)

Animal No.	*Albumin*	*Globulins*		
		α	β	γ
1	42	7	13	38
2	46	3	12	39
3	44	5	13	38
4	48	3	18	31
5	42	3	10	45

area. Edsall (1947) claimed that individual variations in serum protein values are typical of animals. Ewerbeck (1957) has confirmed this, and suggested that these variations are probably due to various sub-clinical conditions. Different breeds of cattle and sheep may have different serum compositions, due to genetic factors.

CONCLUSIONS

Urinary amino-acid excretion in apparently normal cattle and sheep differs from that of other species. As is well known, the predominant free amino-acid is glycine, but paper chromatography shows that other amino-acids occur in the free form and many are in a bound form such as conjugates or peptides.

Electrophoresis of serum proteins in cellulose acetate membranes was used to enable quantitative determination of serum protein fractions in samples from a small number of apparently normal calves and sheep.

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NUCLEAR ABERRATIONS IN CULTURES OF "NORMAL" CELLS FROM FOUR ANIMAL SPECIES

By

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INTRODUCTION

As part of an extensive programme for investigating the visible cellular changes that occur in tissue cultures as a result of the growth of viruses of canine origin, we have studied large numbers of normal cell cultures which were derived from healthy animals of different species. We have found a variety of cytological aberrations which have occurred spontaneously with no apparent relation to the technique and culture medium.

While many workers have described cytological abnormalities in tissue cultures (Kleinfield and Melnick, 1958), these publications are mainly concerned with cultures of either neoplastic cells or normal simian and human cells. A previous communication from this laboratory (Larin, 1958) reported the spontaneous occurrence of intranuclear inclusion bodies of A type (Cowdry, 1934) in cultures of "normal" renal cells from "healthy" dogs. The purpose of this paper is to describe cytological aberrations frequently found in cultures of "normal" cells from "healthy" dogs, pigs, ferrets and chick embryos, and to discuss further the significance of this phenomenon.

MATERIALS AND METHODS

Tissue. Tissue was obtained by aseptic excision either from the living animal under phenobarbitol sodium (nembutal) anaesthesia, or within 30 minutes after euthanasia. Promptly after excision, the tissue was cut into pieces several millimetres in diameter and immersed in Hanks' balanced salt solution. Roller tube cultures were prepared by the basic techniques of Parker (1950). Explants were dissected individually with small stainless steel scalpels from the larger tissue fragments, and were approximately 1 mm. in diameter. The tissue explants were placed in Hanks' BSS until they were incorporated in a thin layer of coagulated chicken plasma on histological coverslips, the latter being accommodated inside thin-walled Pyrex test-tubes (rimless), which were fitted with suitable rubber stoppers. Cultures were incubated at 36° to 37°C until cellular proliferation had become well established. No subcultures of the roller tube cultures were made.

Cultures of trypsin-dispersed kidney cells. Primary cultures were prepared by a modification of the method of Youngner (1954). The cells were grown on histological coverslips inside 1 oz. screw-capped bottles which were incubated in a horizontal position at 36° to 37°C. Only two

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subcultures were undertaken when cell sheets were established (5 to 6 days). During the subculturing the cells were dispersed with 0.5 per cent versene and resuspended in a fresh portion of the medium using the technique of Jordan (1956) to give the desired count.

Plasma and serum. Chicken plasma and serum were prepared from blood which had been aseptically obtained by puncture of the carotid artery in one year old male chickens which had previously been starved for 24 hours and then anaesthetised with nembutal. Plasma was prepared without the use of an anticoagulant and was stored in paraffined tubes. Calf serum was prepared from blood obtained aseptically from the local abattoir. Serum was sterilized by filtering through a Seitz filter. Plasma and serum were kept until use in a refrigerator at 3°C.

Medium. The standard medium comprised 1 per cent lactalbumin hydrolysate and 20 per cent of either chicken or calf serum in Hank's BSS. Phenol red (0.002 per cent), penicillin (100 μ/ml) and streptomycin (100 μg./ml) were added. The medium was sterilized before use by filtering through a Seitz filter. Occasional use was made of the synthetic 199 medium supplied by Glaxo Ltd.

Giemsa staining method. Cultures were fixed in Bouin's fluid for 5 minutes and were kept overnight in 70 per cent ethyl alcohol; the cultures were then washed, stained in an inverted position in dilute Giemsa stain, differentiated and dehydrated in acetone, acetone-xylene and xylene.

Fluorescence microscopy. Cultures were fixed in Carnoy's fluid and then treated with a buffered solution of acridine-orange using the techniques of Armstrong (1956) and Armstrong and Niven (1957). Acridine-orange treated cultures were examined with a Reichert fluorescence microscope (Shandon Scientific Co. Ltd.).

Micrography. Photomicrographs were taken of both Giemsa-stained and fluorochrome-treated cultures. In this paper only micrographs of Giemsa-stained cultures are demonstrated. The micrographs were enlarged when necessary with the use of an "Agiscope" enlarger.

Evaluation of the results. In the evaluation of the experimental data the following were taken into consideration (a) the impossibility of determining accurately the identity of the kind of atypical cells and (b) the difficulty of estimating the incidence of atypical cells. For these reasons no attempt has been made to compare the incidence of cytological aberrations in different cultures, but only those cytological aberrations are described which occurred in at least 2 to 3 per cent of the cell population.

RESULTS

Amoeboid Activity of the Nucleus

As the cells spread out thinly on the coverslip, all the cultures showed simultaneous co-existence of both the protoplasmic continuity and the protoplasmic separation, syncytial structure occurring frequently. With ordinary illumination the cytoplasmic film often appeared to be optically structureless and showed no sharp division between the ectoplasm and the endoplasm; nor could a cell membrane be distinguished in the great majority of separate cells.

With the fluorescence microscope it was possible to identify more structural details; with ultraviolet light activation (3650 A°) many separate cells showed cytoplasmic pseudopodia which were sometimes very long and thin and were thrown out from any side of the cells as if these had been moving in different directions. Nuclear chromatin containing deoxyribonucleic acid emitted a strong yellowish-green fluorescence, whereas ribonucleic acid in the nucleolus was flame red and in the cytoplasm it was crimson red.

The area occupied by the thin film of nuclear substance varied in size and shape in different cells (Figs. 1–9). Most frequently the nucleus was spherical or oval, but every culture had also a large number of nuclei which had either small protrusions into the cytoplasm (Figs. 1–2) or were irregularly shaped and lobed (Figs. 2—9), similar to the nuclei in white blood corpuscles or in the cells of lower organisms, in which the nuclei change their form during different phases of orthogeny and metabolism. Some cell nuclei exhibited highly active writhing or worm-like movements. A typical example of this is shown in Fig. 5 in which can be seen a nucleus which has taken on a coil-like shape. The amoeboid activity of cell nuclei was possibly associated with amitosis and with the formation of polynuclear cells and micronuclei (Figs. 6—9). While mitoses were most numerous during the first two days, the rate of amitosis remained constant until degeneration. In older tissue cultures amitoses were more frequent than mitoses.

Mitotic aberrations were found in all cultures in approximately one third of the dividing cells. The following were particularly common:—tripolar mitoses (Fig. 10), disorganisation of the spindle mechanism (Fig. 11), unequal distribution of chromosomes (Fig. 12) and fragmentation of chromosomes (Fig. 13).

"*Nuclear Phagocytosis*" *and Intranuclear Inclusion Bodies*

The term "nuclear phagocytosis" has been recently introduced by Kleinfield and Melnick (1958) to denote the engulfing of pycnotic cells by the nuclei of "normal" simian cells in vitro. The above authors postulated that the intranuclear inclusions, seen in uninfected cultures of simian tissue cells, may have been related to "nuclear phagocytosis". Simultaneously with the above paper there appeared a preliminary communication from this laboratory (Larin, 1958) which described the occurrence of intranuclear inclusion bodies in uninfected cultures of canine renal cells.

Our findings which relate to the phenomenon of "nuclear phagocytosis" in cultures of different animal cells, may be summarised as follows: all cultures were rich in cellular elements with amoeboid nuclei, some of which were spindle-shaped cells which closely resembled fibroblasts, and others were larger cells, probably of epithelial origin. When the cytoplasm of cells became loaded with phagocytosed material the nuclei of some cells engulfed it after the manner of an amoeba. There was little doubt that

amoeboid nuclei could engulf cytoplasmic material of different chemical compositions. Figs. 14–18 show the "ingestion" of cytoplasmic material by cell nuclei in primary cultures of canine renal cells (Fig. 15) and of chick chorioallantois (Figs. 14, 16, 17, 18). It must be emphasised here that the extensive vacuolation of cells in tissue cultures which can be seen in Fig. 14 was also found in the chorioallantois from 12 day old "normal" eggs. Similar vacuolation was found in cells of chick chorioallantois after inoculation of eggs with vaccinia virus (Kamahora, Sato, Kato and Hagiwara, 1958). Nuclear vacuoles have been found in cultures of human foetus liver cells by Dr. M. Fraccaro, of the State University for Human Genetics, Uppsala, Sweden (1958).

The wrinkling of the thin film of the nuclear substance which can be seen in Figs. 20 and 22 is a phenomenon which was common in cultures of different animal tissues. Comparing Fig. 20 and 22 with Figs. 25–27 it can be concluded that although the wrinkling of the nuclei was probably caused by the movements of the nuclear film, this occurred only in some examples of "nuclear phagocytosis". Figs. 21 and 22 show "phagocytosis" of lymphocyte-like cells by the nuclei of larger cells. In particular, Fig. 21 shows a lymphocyte-like cell in a cytoplasmic vacuole after ingestion, and Fig. 22 a lymphocyte-like cell in the process of being engulfed by the nucleus of the cell-phagocyte. Fig. 23 shows a cell nucleus in the process of engulfing cytoplasmic material, and in Fig. 24 can be seen particles, possibly the nuclear chromatin of a cell, which have been engulfed by the irregular shaped nucleus. The direct relationship of amoeboid activity of the nuclei to nuclear phagocytosis is clear from Figs. 25–27 which shows early and late stages of engulfing of material by the amoeboid nuclei.

Several types of intranuclear inclusion bodies were found in uninfected cultures. (1) Intranuclear inclusions which had the appearance of a cloud-like mass inside the nucleus. This phenomenon was often accompanied by the alteration and displacement of the nucleoli (Figs. 27 and 29). (2) Dark stained concentrations of granular material which probably represented engulfed particles of nuclear chromatin (Fig. 24). This was sometimes vacuolated and displaced towards the periphery, as shown in Fig. 28. (3) Inclusion bodies which fit the description given by Cowdry (1934) to type A inclusion bodies (Figs. 32–35). (4) Inclusions which resembled crystals (Fig. 36).

DISCUSSION

The nature and origin of nuclear aberrations which can occur spontaneously in "normal" animal cells growing in vitro, have not hitherto received adequate attention. Although we do not yet know the mechanism involved in nuclear aberrations this

study indicates that this phenomenon occurs commonly in cultures of "normal" cells from different animal species and that it has no apparent relation to the technique or medium used. Numerous problems are raised by these findings and there are extensive gaps in our knowledge.

It is clear from these studies that nuclear budding, fragmentation and lobation in tissue cultures represent amoeboid activity of the nuclei and that the change of form of the nuclei is not the result of the technique or medium used. An amoeboid nucleus is known to exist in cells of lower animal species, e.g. in the spinning-gland cells of butterflies (Just, 1939). In vertebrates the amoeboid nucleus is normally represented by the polymorphonuclear leucocytes of the blood. Nuclear extrusions in the cytoplasm which represent the initial phase of nuclear fragmentation have long been known to be common in malignant cells in vivo (Howard and Schultz, 1911), and cause the nuclei to become hypertrophied and extensively lobated. In an irregularly shaped nucleus the surface area of nuclear material is greater in relation to the volume of the nucleus than with a spherical nucleus. This may be significant in facilitating nucleo-cytoplasmic exchanges in vivo and in vitro. It is not yet known in what respects cultures of "malignant" cells in vitro differ from cultures of "normal" cells.

Even more striking is the presence of intranuclear inclusion bodies. There is no escape from the conclusion that a relationship exists between "nuclear phagocytosis" and intranuclear inclusions, and perhaps a hint about what may happen in vivo is given by this study. The nature of the phagocytosed material and its biological effect on the nucleus is far from clear, but the study conducted by Kleinfield and Melnick (1958) and the present investigations suggest that a clearer conception of the nature and activity of the nucleus is beginning to emerge, even though great gaps still exist in our knowledge and much of the story concerning nuclear functions is at present conjectural.

Although the occurrence in vivo or in vitro of such nuclear abnormalities as intranuclear inclusion bodies is often associated with virus activity we do not know whether these abnormalties are truly induced by virus or are merely associated with the nuclear activity it causes. The incidence of mitotic aberrations was high in our cultures and they included great variation in shape of chromosomes, multipolar spindles, scattered chromosomes without attachment to the spindle, and unequal distribution of chromosomes and failure of some of them to separate.

CONCLUSIONS

A high incidence and great variety of cytological aberrations were observed in short term cultures of normal cells from healthy

dogs, pigs, ferrets and chick embryos. They bore no apparent relation to either the technique or culture medium.

The most striking phenomenon was amoeboid activity of cell nuclei, the incidence of which was high in all the cultures studied. It is suggested that amoeboid nuclei may be responsible for the phenomenon of "nuclear phagocytosis" and also for the spontaneous occurrence of intranuclear inclusion bodies.

ACKNOWLEDGMENTS

The authors are indebted to Dr. W. R. Wooldridge for his interest and support and to Dr. J. A. Armstrong and Dr. Janet Niven for invaluable information concerning their method of histochemical differentiation of nucleic acids by means of induced fluorescence.

Capable technical assistance which was given by Misses M. K. Baker and J. Gillingham, also by Messrs. D. Norton and R. Watt, is acknowledged with pleasure.

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Plate I

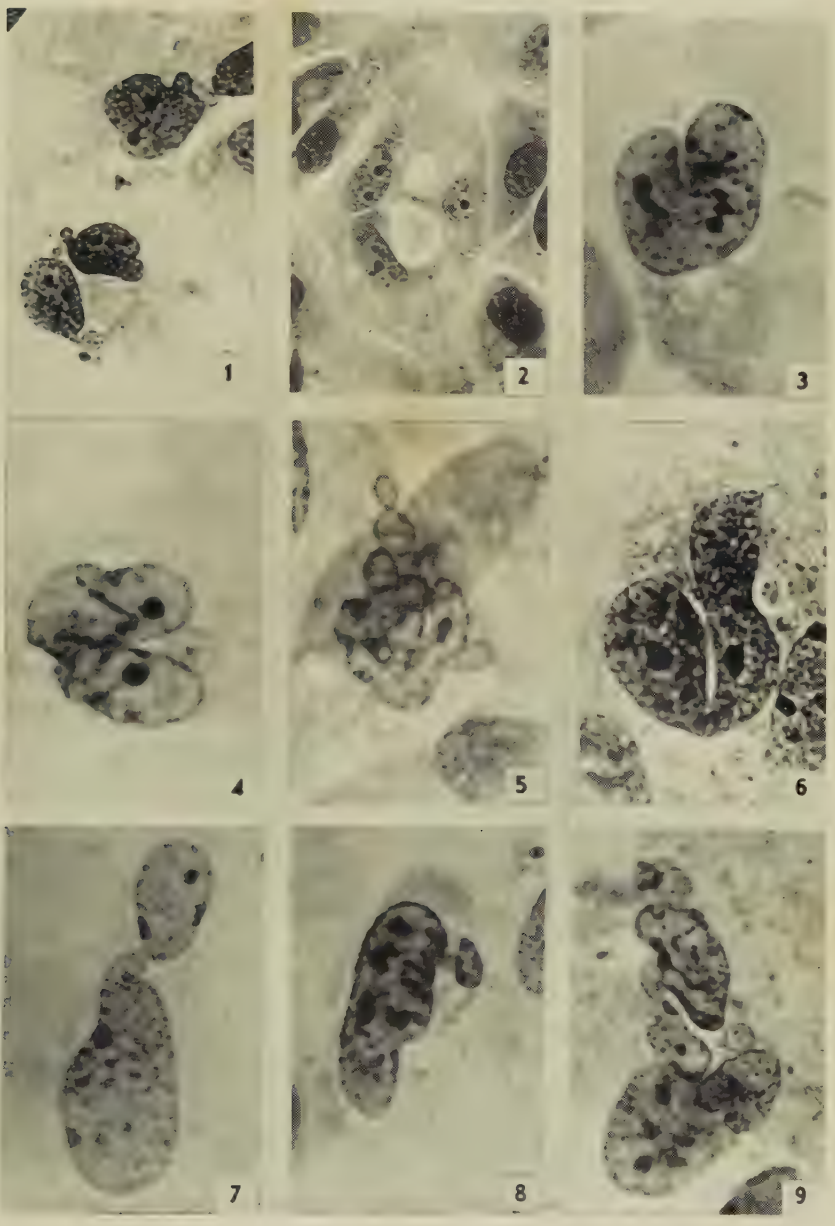

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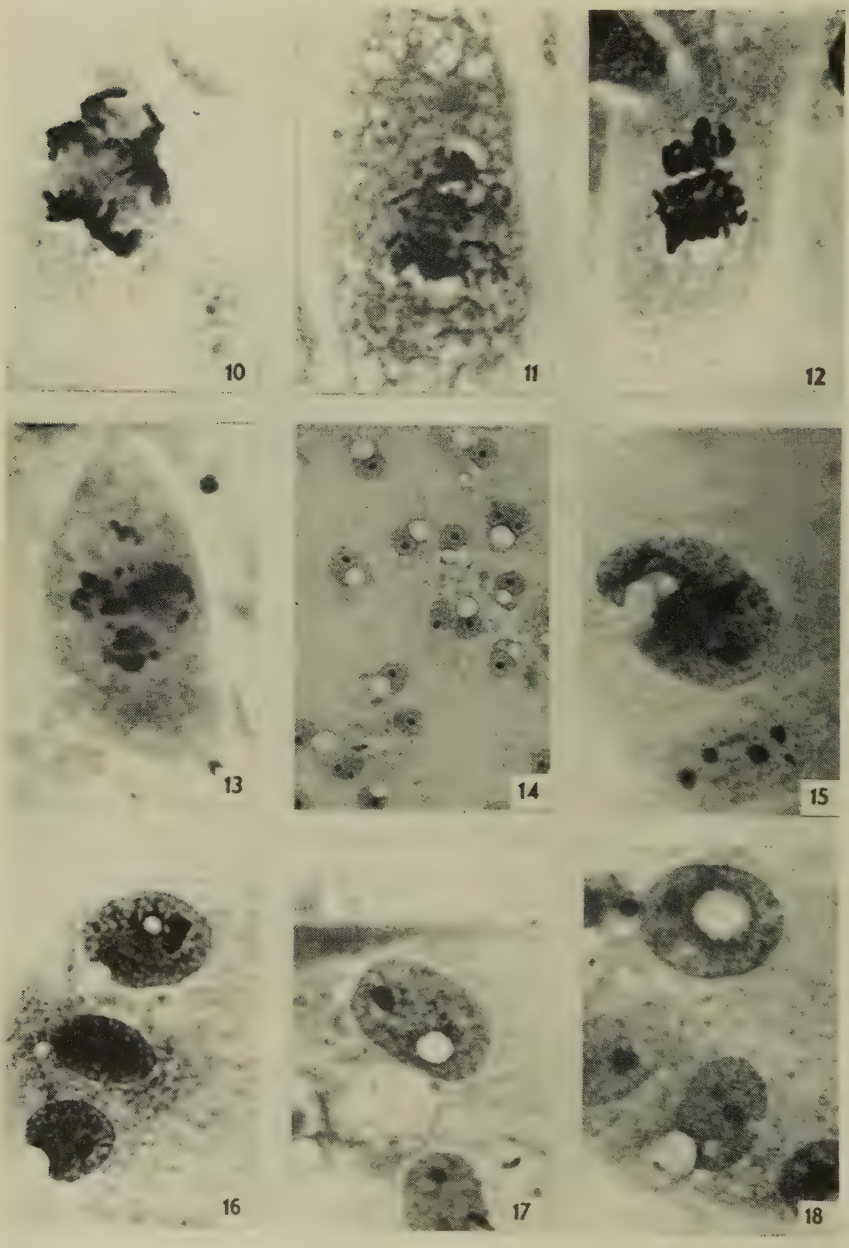
Plate II



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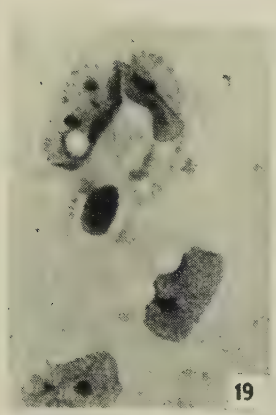

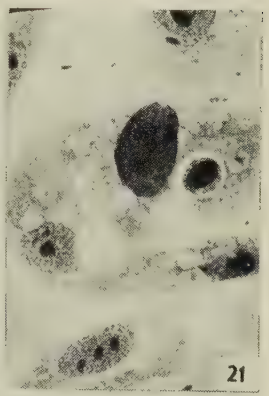

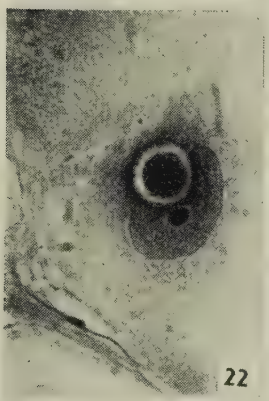

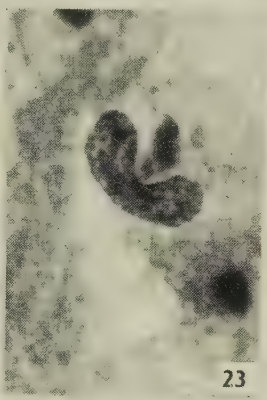

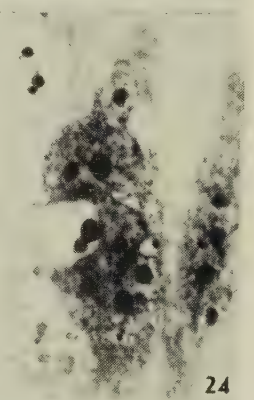

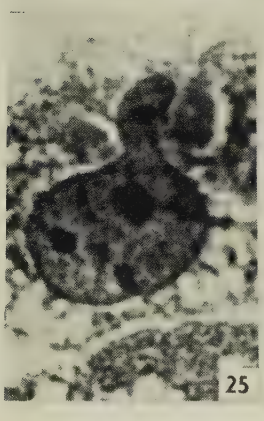

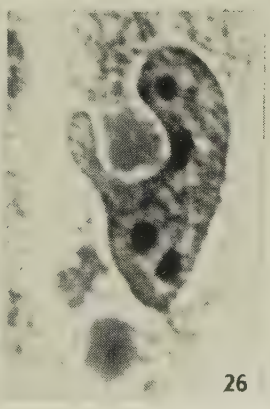

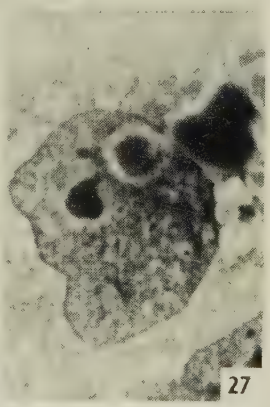

Plate IV

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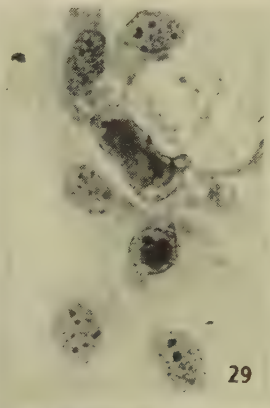
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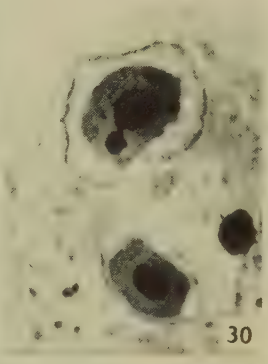
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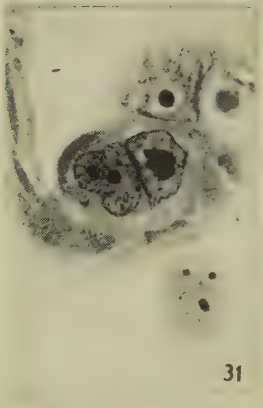
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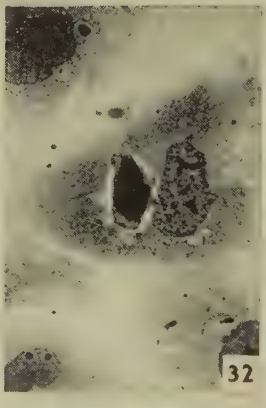
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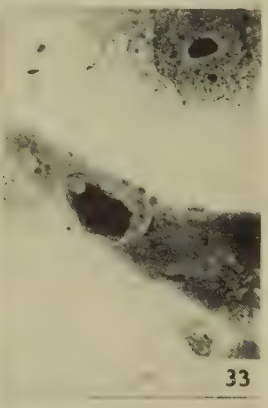
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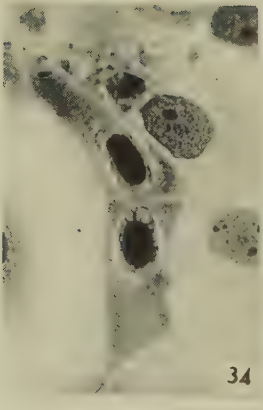
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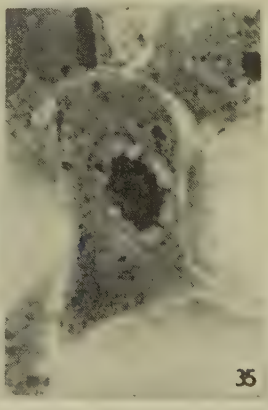
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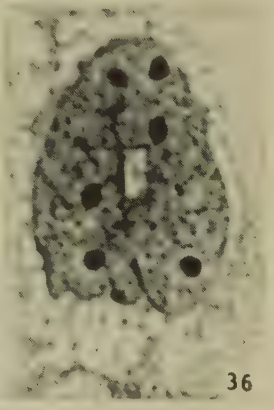
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LEGENDS

Plate 1

Figs. 1 and 2. Amoeboid nuclei in giant cells from a primary culture of adult pig kidney. (Trypsin-dispersed). Note the protrusions coming from the nuclei.

Fig. 3. Lobation of a cell nucleus in a primary culture of embryonic ferret kidney. (Roller-tube,).

Fig. 4. Extensive lobation of a nucleus in a primary culture of embryonic ferret liver. (Roller-tube,).

Fig. 5. A coil-like nucleus. Primary culture of embryonic ferret kidney. (Roller-tube,).

Fig. 6. Amoeboid nucleus in a cell from a primary culture of adult pig kidney. (Trypsin-dispersed,).

Figs. 7 and 8. Amitosis in a primary culture of embryonic ferret liver. (Roller-tube,.)

Fig. 9. Amoeboid nuclei in a primary culture of embryonic ferret kidney. (Roller-tube,).

All × 700.

Plate 2

Fig. 10. A tripolar anaphase with lagging chromosomes in a cell from a primary culture of adult pig kidney. (Trypsin-dispersed, × 700).

Fig. 11. Disorganisation of the spindle mechanism in a primary culture of adult dog kidney. (Trypsin-dispersed, × 700).

Fig. 12. Mitosis in a cell from a primary culture of adult pig kidney. Disorganisation of the spindle mechanism. (Trypsin-dispersed, × 700).

Fig. 13. Disorganisation of the spindle mechanism and breaking of chromosomes in a cell from a primary culture of adult dog kidney. (Trypsin-dispersed, × 700).

Fig. 14. Primary culture of chick chorioallantois. Cells show extensive vacuolation of cytoplasm and nuclear change. (Roller-tube, × 130).

Fig. 15. Invagination of the nucleus encircling cytoplasmic material. Primary culture of adult dog kidney. (Trypsin-dispersed, × 700).

Figs. 16, 17 and 18. Large nuclei containing commonly seen inclusions. These appear to have been formed by an invagination of the nuclear film encircling cytoplasmic material. Primary culture of chick chorioallantois. (Roller-tube, × 700).

Plate 3

Fig. 19. Nuclear inclusion which appears to form in association with an invagination of nuclear material. Primary culture of chick chorioallantois. (Roller-tube, × 700).

Fig. 20. Nucleus with sharply delineated bands, probably caused by wrinkling of the nuclear film. Primary culture of chick chorioallantois. (Roller-tube, × 700).

Figs. 21 and 22. Phagocytosis with nuclear involvement. In Fig. 22, the nucleus is encircling the phagocytised (lymphocyte-like) cell. Primary culture of adult dog kidney. (Trypsin-dispersed, × 350).

Fig. 23. "Nuclear phagocytosis" in a cell from a primary culture of chick chorioallantois. (Roller-tube, × 700).

Fig. 24. "Nuclear phagocytosis" in a cell from primary culture of adult dog kidney. Note chromatin—like material which has been engulfed by the nucleus. (Trypsin-dispersed, × 700).

Figs. 25, 26 and 27. Different stages of "nuclear phagocytosis" in cells from a primary culture of adult dog kidney. The nuclei are engulfing cytoplasmic material. (Trypsin-dispersed, × 700).

Plate 4

Fig. 28. A large nucleus containing a commonly seen vacuolated inclusion. Primary culture of chick chorioallantois. (Roller-tube, × 700).

Figs. 29 to 36. Commonly seen intranuclear inclusion bodies in canine renal cells, second subculture. (Trypsin-dispersed, × 700).

FURTHER STUDIES ON THE EXPERIMENTAL TRANSMISSION OF AVIAN OSTEOPETROSIS

By

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INTRODUCTION

In a previous paper (Holmes, 1958) it was shown that avian osteopetrosis could be transmitted to normal fowls by the intraperitoneal inoculation into day old chicks of whole blood, bone marrow or unfiltered plasma from birds with the active disease. The effect of filtered plasma is now described. An experiment was also carried out to determine the infectivity of unfiltered plasma when administered by mouth to day old chicks. It was also shown that the disease could be induced by injection of blood into the amnion of the developing chick embryo and it was considered that the egg might be one natural mode of transmission. This was further investigated. Using artificial insemination, affected females were inseminated with semen from affected males and the resulting progeny were reared to 6 months of age. The results of this experiment are recorded.

It was observed in earlier experiments that a little less than half the inoculated chicks subsequently developed osteopetrosis. Whether the susceptibility is related to genetic factors or to the development of an immune response is not known. Cortisone and hydrocortisone have been shown to decrease antibody production in rabbits (Bjorneboe, Fischel and Stoerk, 1951; Germuth, Oyama and Ottinger, 1951; Kass, Kendrick and Finland, 1955), and A.C.T.H. and cortisone or cortisone alone increased the susceptibility of mice and hamsters to poliomyelitis virus (Schwartzman, 1950, 1952). Similarly in experimental infection with *M. tuberculosis,* cortisone favoured the spread of the disease in guinea pigs (Spain and Molmut, 1950) and rats (Michael, Cummings and Bloom, 1950). As far as the writer is aware the influence of cortisone on resistance to infections in the fowl is not known. Observations were therefore made to see if cortisone administration would alter the susceptibility of chicks to experimental osteopetrosis.

MATERIALS AND METHODS

The materials used for transmission were plasma, both filtered and unfiltered: whole blood was also inoculated for purposes of comparison. The whole blood and plasma were obtained as described previously (Holmes, 1958). Plasma was filtered through a Seitz EK filter immediately after centrifugation and the filtrate was inoculated intraperitoneally into day old chicks in a dose of 0.5 ml. Unfiltered plasma was

given per os in a dose of 0.3 ml. or 0.5 ml. repeated at 1, 2 and 3 days after hatching. In the intervals between dosing it was stored at 4°C.

As in the previous experiments, day old "as hatched" chicks were obtained from a commercial hatchery and a variety of breeds and crosses was used, except in the later experiments when only RIR x LS males were inoculated. Control, uninoculated chicks were kept as a check against the possibility of the introduction of naturally infected birds. Both control and inoculated birds were kept for at least six months before slaughter. At postmortem leg and wing bones were X-rayed to detect any signs of osteopetrosis. Previous experiments having indicated that 35 days of age was the earliest time at which signs of the disease appeared, chicks dying under one month of age were not considered in the evaluation of the experiments. The donors used had all been experimentally infected when one day old by intraperitoneal inoculation of whole blood or filtered plasma from active cases of the disease. They were all markedly affected, with visible thickening and increased heat over the metatarsi.

Semen was collected from two experimentally infected males showing typical signs of osteopetrosis and five affected pullets were inseminated. A dose of 0.1 ml. of undiluted semen was used, insemination being carried out immediately after collection and repeated every 3 to 4 days. These birds were housed in a battery and fed on breeders pellets. The eggs were incubated in four separate batches and the resulting chicks were reared to 6 months of age.

For the cortisone experiment four batches of 20 RIR x LS day old male chicks were obtained. One batch was left as a control while another received cortisone acetate only. The third batch was given whole blood intraperitoneally and the fourth group received whole blood and cortisone acetate. The cortisone acetate was given daily by intramuscular injection from 1 day old, for a period of 23 days. The dose used varied from approximately 6 mg/kg at 1 day old gradually increasing to 6.2 mg/kg at 8 days and 57.8 mg/kg at 22 days of age.

TABLE 1

THE INFECTIVITY OF 0.5 ML. OF FILTERED PLASMA BY INTRAPERITONEAL INJECTION IN DAY OLD CHICKS

Donor No.	*Injected birds*			*Control birds*		
	Total	*Surviving 1–6 mths.*		*Total*	*Surviving 1–6 mths.*	
		Osteopetrosis	*No effect*		*Osteopetrosis*	*No effect*
45	19	0	19	8	0	8
1103	14	4	8	10	0	10
1475	22	6	14	20	0	20
Total	55	10	41	38	0	38

RESULTS

Infectivity of Filtered Plasma by Intraperitoneal Injection

The results of three experiments using filtered plasma are shown in Table I. In the first experiment no cases of osteopetrosis developed, but the disease appeared in the other two. No cases occurred in the controls.

For comparative purposes whole blood from donor 1103 was inoculated into day old chicks at the same time as the filtrate experiments. From 15 chicks injected seven birds developed osteopetrosis.

TABLE 2

INCIDENCE OF OSTEOPETROSIS IN THE PROGENY OF AFFECTED MALES X AFFECTED FEMALES (ARTIFICIALLY INSEMINATED)

Dam No.	*Sire 154*		*Sire 762*	
	No. of chicks surviving 1 mth.	*No. of cases of osteopetrosis*	*No. of chicks surviving 1 mth.*	*No. of cases of osteopetrosis*
164	10	2	7	1
57	4	1	–	–
70	6	1	1	0
27	3	0	5	0
1102	17	0	4	0
Total	40	4	17	1

TABLE 3

THE INFLUENCE OF CORTISONE ACETATE ON SUSCEPTIBILITY TO OSTEOPETROSIS

Donor No. 1493 All chicks RIR x LS

Treatment	*Total*	*Birds surviving 1–6 mths.*	
		Osteopetrosis	*No effect*
Controls	20	0	20
Controls + Cortisone i/m	20	0	18
0.5 ml. whole blood i/p ..	20	9	10
0.5 ml. whole blood i/p + Cortisone i/m ..	20	8	12

Routes of Natural Transmission

Through the egg. Following successful transmission of osteopetrosis by intra-amnion inoculation as reported previously the effect of

artificial insemination of affected females with semen from affected males was studied. The results, using two males and five females, are shown in Table 2.

By ingestion. Six chicks were dosed at one, two and three days of age with 0.3 ml. of unfiltered plasma from donor 1103 and four chicks similarly received 0.5 ml. per day for 3 days. Of these birds two in the latter group subsequently developed osteopetrosis.

Influence of Cortisone on Susceptibility

The effect of repeated doses of cortisone acetate given intramuscularly for the first 23 days of life on the susceptibility of day old chicks to intraperitoneal injection with material from an active case of osteopetrosis is shown in Table 3.

DISCUSSION

The results show that the disease may be transmitted by filtered plasma, indicating that the infective agent is of small particle size, probably a virus. In the first experiment using filtered plasma, no cases of osteopetrosis developed but in two subsequent experiments 33 and 30 per cent. of birds, respectively, were affected. In previous experiments (Holmes, 1958) 46 per cent. of 52 birds inoculated with whole blood developed osteopetrosis and whilst the numbers in this study are small the results indicate that filtered plasma may not be as infective as whole blood. The results using unfiltered plasma by mouth show that the oral route is also effective in transmitting the disease in early life.

Of 57 chicks produced by artificial insemination from affected parents five birds developed osteopetrosis. This incidence is low (8.8 per cent.) but it is considered that these results provide further evidence that the egg may be one route of natural transmission of this disease. If this is so then in the field one must assume the existence of carriers, since clinically affected birds would obviously not be used for breeding purposes.

Cortisone acetate failed to influence the incidence of osteopetrosis in these experiments, the results being similar to those obtained with whole blood alone. The effect of cortisone in the bird may differ from that observed in some mammals, however, and this apparent lack of effect of cortisone does not necessarily rule out the possibility that an immune response may explain the failure of a fairly high proportion of inoculated birds to develop osteopetrosis.

CONCLUSIONS

Experiments are described which show that the agent producing avian osteopetrosis when inoculated intraperitoneally into day old chicks, will pass through a Seitz EK filter. The disease also

occurred following oral administration of unfiltered plasma. A small percentage of the progeny of affected parents also developed the condition.

Cortisone acetate given intramuscularly every day for the first 23 days of life following inoculation with whole blood at one day old appeared to have no influence on the incidence of the disease.

ACKNOWLEDGMENTS

I wish to thank Professor C. S. G. Grunsell for his interest and for his help in the compilation of this paper and Miss A. Sparey, Miss P. Pockson and Mr. R. Eglon for technical assistance.

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THE NORMAL HAEMOGRAMS AND COAGULOGRAMS OF THE ENGLISH THOROUGHBRED HORSE

By

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INTRODUCTION

For the purposes of this article, the word haemogram indicates the cellular status of peripheral blood, while coagulogram indicates the coagulability of blood as measured by some of the newer techniques. These terms are used for convenience to express these two characteristics of the total blood picture.

There have been a number of published reports on the cellular blood picture of Thoroughbreds from the U.S.A. but only very limited observations in this country and none from the traditional English Thoroughbred centre, Newmarket. It is difficult to collect a large series of blood specimens from clinically normal animals since owners and trainers are naturally reluctant to subject them to unnecessary interference. It is even more difficult in the case of stallions which are at stud and often of very great value.

In the last three years the Equine Research Station has had an opportunity to examine 132 blood specimens from clinically normal animals of all ages and types, both in and out of training. The first part of this paper analyses the haemograms of these animals. The determination of coagulograms is limited by the necessity to perform some of the examinations at once in the laboratory ; it is therefore required that subjects for investigation must be at the laboratory premises. The normal range of the newer coagulation tests which we have applied to Thoroughbreds is quite narrow and is therefore offered with confidence although it is based upon a relatively small sample of the Thoroughbred population.

Horses other than Thoroughbreds have been excluded from consideration in this paper, since we have confirmed the previous findings of others that their haemograms are widely divergent from the norm for the Thoroughbred.

MATERIAL AND METHODS

Collection of specimens. Collections were always made from the jugular, the vein being raised by digital pressure. Local anaesthesia of the overlying skin was used in excitable animals. The blood was withdrawn by a 20 or 22 S.W.G. stainless steel needle, sterilised by hot air, into a sterile, paraffined all-glass syringe. Samples for haematological investigation were transferred immediately to a siliconised 5 ml. bijou bottle which contained 1.1 mg of the di-K salt of ethylene-diamine-tetra-acetic acid ("sequestrene") in the dry state. The bottles were prepared by adding 0.1 ml. of an 11 per cent solution of the salt and

evaporating to dryness at 50°C. Specimens of plasma for coagulation were obtained by mixing 1 ml. of 3.8 per cent trisodium citrate with 9 ml. of whole blood in a siliconised centrifuge tube and centrifuging at 3,000 r.p.m. for 20 minutes. Serum was obtained by allowing about 10 ml. of blood to clot in glass and incubating it for 4 hours at 37°C. The serum was recovered by centrifugation. For the estimation of whole blood clotting time, 1 ml. amounts of blood were delivered from the syringe to dry 3″ x $\frac{3}{8}$″ glass test tubes (not siliconised).

Laboratory procedure for the haemogram. Specimens were always examined with minimal delay, never more than 4 hours after taking the specimen. Bottles were shaken in a mechanical device (Elliott, Liverpool), for one minute and dilutions were made during the next 90 seconds only. If more time was required for any reason, the bottle was reshaken. Blood for haemoglobin estimation was taken up in a 0.05 ml. constriction type micropipette ("E-Mil" type) and expelled into 20 ml. of 0.04 per cent ammonia. Solutions were read at 540 mμ on a Unicam SP1400 absorptiometer. Erythrocyte counts were made by dilution in Hayem's fluid, counting being performed on Spencer AO "Bright Line" haemocytometers. Erythrocyte sedimentation rates were measured by a single reading at one hour of specimens in standard Wintrobe haematocrit tubes. These tubes were subsequently centrifuged at 3,000 r.p.m. for 30 minutes to obtain the packed cell volume.

Total leucocyte counts were made by dilution in a standard acetic acid and gentian violet fluid, while eosinophils were assayed by dilution in the fluid of Discombe (1946); both were counted in "Bright Line" haemocytometers. Smears were made with a mechanical device (Marks, Bailey and Gunz, 1950) and stained with Leishman stain (Hopkins and Williams) diluted in phosphate buffer pH7.4. Differential counts were made on 200 cells and from these and the total leucocyte counts, absolute counts of the various cell types per c.mm. were calculated.

Smears were also stained to demonstrate leucocytic alkaline phosphatase by the method of Kaplow (1955). This enzyme is confined to the granulocytes and a "score" for a particular animal can be obtained by examining 100 granulocytes on the smear and assaying each for enzymic activity on a O to ++++ basis. Theoretic score limits are, therefore, 0 to 400. In our hands and with equine material Fast Blue RR is a preferable diazotised stain to the Fast Garnet recommended by Hayhoe and Quaglino (1958). These procedures were very carefully standardised and carried out by trained technicians whose work was checked and found to be repeatable with only minor variations.

Laboratory procedure for the coagulogram. Whole blood clotting times were determined by the method of Lee and White (1913) by inverting at least 2 test tubes 3″ x $\frac{3}{8}$″, containing 1 ml. of blood, every half minute in a water bath at 37.5°C commencing within one minute of obtaining the blood from the patient, until solid clots were formed. One-Stage prothrombin times were performed according to the method of Quick (1938) using thromboplastin prepared from horse brain. The test was also performed using Russel Viper Venom ("Stypven", Burroughs Wellcome and Co.) as a thromboplastic source both with and without added lipid in the form of a chloroform extract of brain. Clot retraction was estimated by the method of Macfarlane (1939). Thromboplastin

generation was measured by the test of Biggs and Douglas (1953) using the chloroform extract of brain of Bell and Alton (1954) to replace platelets. All dilutions were made in buffered normal saline, using glyoxaline buffer pH 7.35 (Mertz and Owen, 1940) in place of water as a diluent. Platelet counts were made by dilution from the sequestrenised blood sample in 1 per cent disodium ethylene-diamine-tetra-acetic acid in normal saline.

RESULTS

Haemogram

An analysis of the available results indicated that reasonable numbers in each class would be obtained by division into birth to 6 months old, two years old, geldings over 2 years, and mares over 2 years. As there were only two Stallions they are excluded from consideration. It also became evident that training had no effect on the haemogram provided the specimens were taken at rest and without exciting the animal. As has been reported previously (Scheunert and Krzywanek, 1926; Holman, 1947; Brenon, 1958) any excitement of a horse leads to a dramatic increase in red blood cell values in two or three minutes. In this study care was taken to avoid any excitation of the animals and those which were unavoidably excited were rejected.

The results are presented in Table 1 where the four groups mentioned are given separately, and an overall set of figures is also derived. It will be seen that there is a decrease in erythrocyte count as the age of the animal increases, but this is not accompanied by appreciable change in haematocrit or haemoglobin levels. The MCHC is somewhat lower in the youngest age group than it is in the remainder. Erythrocyte sedimentation is approximately constant with very wide ranges in all groups. This test appears to be particularly variable with horse blood and is certainly markedly influenced if anticoagulants other than sequestrene are used, if the laboratory temperature varies more than 3 or 4 degrees centigrade, or if the blood is stored for more than about 4 hours before testing.

The total leucocyte numbers do not vary greatly with age, but there is some decrease of neutrophils and increase of lymphocytes at maturity. There is also some increase of eosinophils with age which may be linked with the parasitic burden of the subjects.

Leucocytic alkaline phosphatase activity is reduced in foals less than seven days old. The distribution of activity is shown in Fig. 1 and Table 3. No normal equine bloods have been seen which gave a score greater than 350 or less than 50.

Coagulogram

Table 2 sets out the range of normal results obtained from some standard blood coagulation tests. Twelve animals were used which were clinically and haematologically normal. The small

TABLE 1

NORMAL THOROUGHBRED BLOOD VALUES

Category		Foals *Birth—6 mths.*			*Two-year-olds*			*Geldings*			*Mares*			*Overall*		
Number examined		*38*			*36*			*34*			*24*			*132*		
		Mean	*Range*	*S.D.*	*Mean*	*Range*	*S.D.*	*Mean*	*Range*	*S.D.*	*Mean*	*Range*	*S.D.*	*Mean*	*Range*	*S.D.*
Haemoglobin		14.1	10.5–19.2	2.4	14.9	11.8–17.8	1.4	15.0	11.0–17.8	2.2	14.5	10.7–18.3	2.9	14.7	10.5–19.2	0.94
Erythrocytes $x10^6$		10.2	7.0–13.1	0.4	9.5	7.3–10.8	2.1	9.4	7.5–11.4	1.76	8.0	6.23–9.95	2.1	9.45	6.23–13.1	1.11
Haematocrit		41.6	31.0–52.0	8.5	40.7	34.5–46	5.2	42.0	33.5–56.0	5.1	40.3	30.0–57.0	6.1	41.2	30.0–57.0	6.6
M.C.H.		13.7	10.8–17.7	1.6	16.1	12.4–19.1	1.7	16.7	13.7–19.9	2.0	18.2	13.6–20.6	2.3	15.9	10.8–20.6	2.64
M.C.V.		42.0	30.7–48.3	5.2	44.3	36.9–54.8	3.6	46.8	37.1–54.0	3.3	50.6	35.1–60.5	6.6	45.2	30.7–60.5	5.32
M.C.H.C.		33.3	30.0–39.0	4.7	36.2	29.8–40.6	5.7	35.7	32.7–39.5	1.8	36.1	30.5–39.6	2.8	35.1	29.8–40.6	3.6
E.S.R.		32.0	1–61	18.5	45.0	22.0–57	13.1	50.8	19–66	10.4	34.5	0–59	20.9	40.1	1–61	13.2
Leucocytes		8360	4500–12950	3300	9330	6220–13400	2070	7040	4740–11525	2750	8450	5050–12050	1560	8420	4500–13400	2220
Neutrophils	Juveniles	28.6	0–272	15.4	8	0–268	14.5	3	0–58	12.1	0	0	–	11.4	0–272	9.1
	Stab	426	114–1360	114	224	0–1610	186	168	0–1180	208	190	0–660	157.8	260	0–1610	23.1
	Segmented	6050	2650–9950	1786	4890	2620–8800	1612	4640	2350–8450	1688	5200	2780–8410	1640	5225	2780–9950	523
Eosinophils		62	0–780	65.9	169	31–870	68	204	0–765	81.1	232	0–598	57.4	151	0–870	212
Direct Eosinophils		17.6	0–322	11.4	161	0–483	34.1	172	0–1044	85.7	235	11–750	42.9	137	0–1044	185
Basophils		1.9	0–43	8.2	20	0–125	30.7	24	0–102	33.2	22	0–96	37.4	16	0–125	9.1
Lymphocytes		2082	845–4860	864	3650	2050–6170	1341	2500	1330–3920	606	2520	1430–4950	733	2700	845–6170	531
Monocytes		210	0–665	71.8	294	0–925	66.9	252	0–655	51.4	235	30–550	47.5	248	0–925	48.2

Table 1—*Continued.*

NOTES: Haemoglobin as g. per 100 ml. Erythrocytes x10^6 per c.mm. Haematocrit %. E.S.R. mm. per hour. Leucocytes etc. all as numbers per c.mm. "Juveniles", very immature neutrophils with "Kidney bean" shaped nucleus. "Stab", immature neutrophils with "Staff-like" or unlobed nucleus. "Segmented", fully adult neutrophils with lobed nucleus. "Eosinophils", count derived by calculation from the differential and total leucocyte counts. "Direct eosinophils", count made by direct dilution in Discombe's fluid.

size of the group of Thoroughbreds examined did not allow of subdivision for age or sex, but, from this small series, no differences were apparently referable to these factors. Observations upon the newborn foal have not yet been completed.

The coagulogram of the normal Thoroughbred has not been the subject of a specific study. Various papers have reported on the coagulation mechanism of the horse (Archer and Flute, 1959; Bell, Tomlin and Archer, 1955; Barkhan, Tomlin and Archer, 1957; Fantl and Marr, 1958), but ranges for normal Thoroughbreds

TABLE 2

NORMAL COAGULOGRAM OF THOROUGHBREDS

Platelets		90—170,000 per cu.m.
Lee White clotting times		6½—9 minutes.
1-Stage prothrombin times	Horse brain thromboplastin	11—15 secs.
	Russel Viper venom	16—20 secs.
	Russel Viper venom + Cephalin	7— 9 secs.
Clot retraction		45%—60%.

TABLE 3

NORMAL THROMBOPLASTIN GENERATION OF THOROUGHBREDS

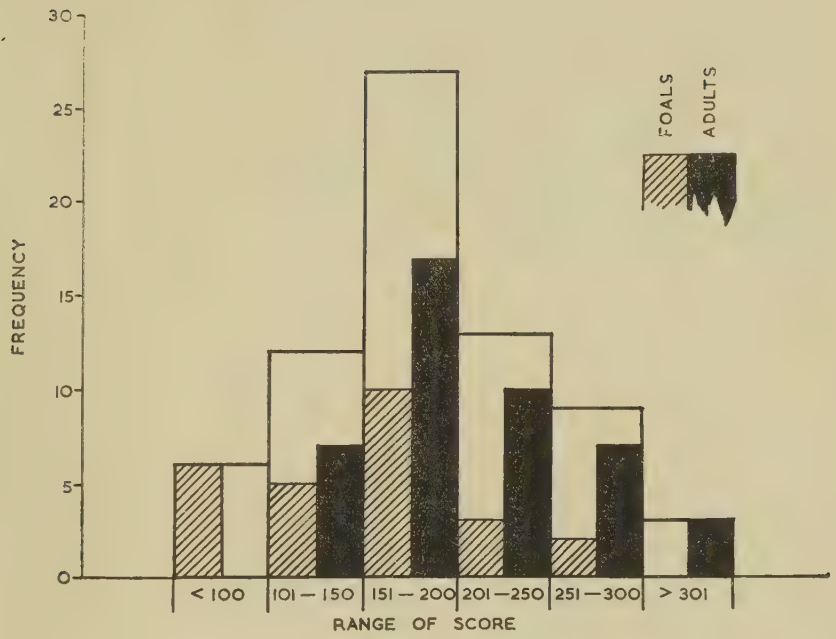
Calibration curve

Dilution of thromboplastin %	*100*	*90*	*80*	*70*	*60*	*50*	*40*	*30*	*25*	*20*	*15*	*10*	*5*
Clotting time of substrate in secs.	12	12.5	13	13.5	14	14.5	15	17.5	20	25	29	36	90

Typical test result

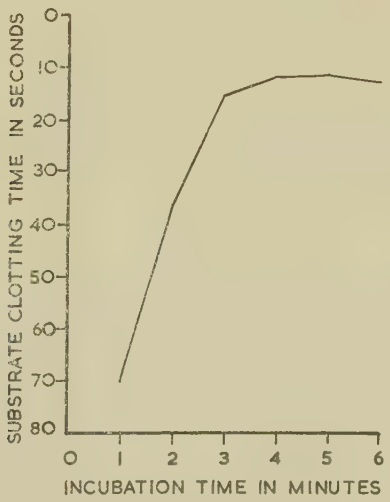
Incubation time mins.	*1*	*2*	*3*	*4*	*5*	*6*
Substrate clotting time secs.	70	36	15	11	11	12
% Thromboplastin	6	10	40	110	110	100

Fig. 1.



Leucocytic alkaline phosphatase activity. The distribution of the alkaline phosphatase "score" of the neutrophils of 71 normal Thoroughbred horses. "Foals": birth to seven days of age. "Adults": Thoroughbreds over 6 months of age.

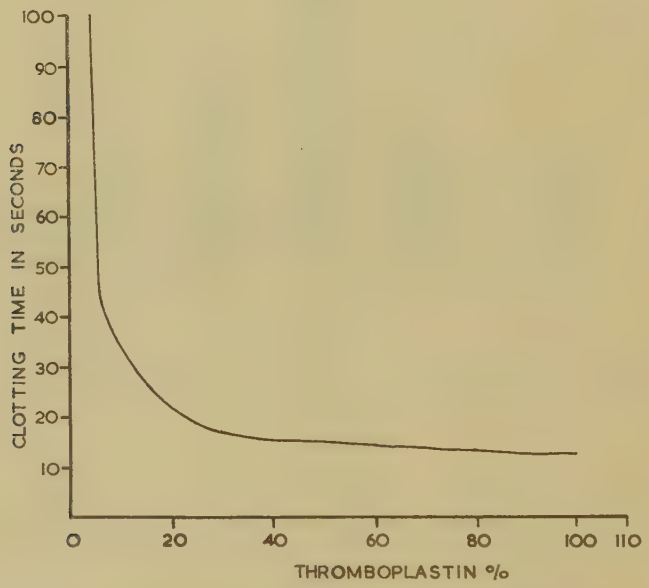
Fig. 2.



Thromboplastin generation in the Thoroughbred. Chloroform extract of horse brain to replace platelets.

were not given. Fig. 2 shows a typical result of a thromboplastin generation test in the Thoroughbred. Fig. 3 illustrates a calibration curve, based wholly upon equine material, relating the concentration of formed thromboplastin to substrate clotting time for use in this test. The curve is obtained by diluting formed blood thromboplastin to a known extent at 0°C. and then estimating the clotting times of substrate plasma aliquots. The shortest time obtained (12 seconds) is assumed to be 100 per cent. Fig. 4 shows the same result as Fig. 2 but plotted as a percentage of thromboplastin by using the calibration curve.

Fig. 3.



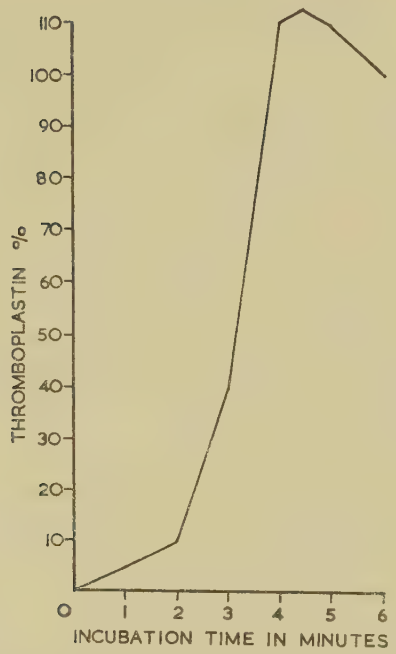
Thromboplastin calibration curve. Dilutions of formed thromboplastin are added to substrate plasma and the clotting times plotted using horse reagents throughout. The shortest clotting time obtained, with undiluted thromboplastin, is assumed to be 100 per cent.

TABLE 4

LEUCOCYTIC ALKALINE PHOSPHATASE ACTIVITY IN THOROUGHBREDS

Age group	*Number examined*	*Mean*	*Range*	*S.D.*
Foals (birth to one week) ..	26	156	46–300	19.9
Other Thoroughbreds ..	45	213	102–390	19.6
Overall	71	192	46–390	21.6

Fig. 4.



Thromboplastin generation in the Thoroughbred. The same curve shown in Fig. 2 but plotted as percentage of thromboplastin against incubation time by the use of the calibration curve shown in Fig. 3.

DISCUSSION

An exhaustive haematological investigation of Thoroughbreds was undertaken by Hansen and his colleagues of Kentucky. Hansen, Todd, Kelly and Cawein, 1950, published a monograph and a series of papers (Hansen, Todd and McGee, 1950; Hansen, Todd, Kelly and Hull, 1950; Hansen, Todd, Kelly, Cawein and McGee, 1950; Hansen, Todd, Cawein and McGee, 1950; Hansen, Todd, Kelly and Cawein, 1951), in which groups of 60 or more animals were classified as mares in foal, barren mares and weanlings. The group of stallions was of 36 only. Todd, McGee, Wyant and Hollingsworth (1951) published a comparable report on sucklings from the same laboratory. It is not possible to make a strict comparison between the figures of these workers and our own, because our results are somewhat differently expressed and because our groups were much smaller and differently constituted. The general impression gained, however, is that their figures for erythrocyte counts are somewhat higher than ours while their haemoglobin determinations are rather lower. Haematocrits are about the same. The work of MacLeod, Ponder, Aitken and Brown (1947) is in general agreement with the figures of Hansen and his colleagues.

Brenon (1956, 1958) published average figures obtained from an examination of 359 clinically normal Thoroughbreds bled prior to racing in California and based upon specimens containing "1 drop of 1.5 per cent lithium oxalate per millilitre of blood." His results are comparable to ours and those of Hansen and his colleagues so far as haemoglobin (14 g. per cent) and P.C.V. (43 per cent) are concerned, but his erythrocyte level is considerably lower (6.9×10^6 per c.mm.). There have been a number of other published reports on the cellular characteristics of horse blood (Holman, 1947; Stewart and Holman, 1940) but they consist of samples essentially from breeds other than Thoroughbred and are, therefore, not strictly comparable with the results reported in this paper.

The haemograms given for Thoroughbreds are of importance as a basis for interpreting results obtained by comparable methods in horses suffering from both inapparent sub-clinical conditions and from frank clinical abnormalities. It should be emphasized that some experience is needed in assessing the exact significance of a set of results where one or two findings are outside the normal ranges, but where the remaining results are found to be within normal values. These and other relevant details have been indicated in another publication (Archer and Miller, 1959).

Some of the ranges quoted in Table 1 are very wide, especially for the leucocyte figures. These are caused by one or two very divergent figures from horses which were clinically normal, but haematologically abnormal in a single respect. These cases were not excluded and it is therefore suggested that the mean figures, considered with the Standard Deviation for each, give a more accurate picture of normality than can be obtained from the ranges alone. An excellent example of this is given by the "Direct Eosinophils" figure in Table 1 for "Geldings" where the range is 0 to 1044, the mean is 172 and the S.D. 85.7. Only one animal in fact exceeded 260 eosinophils per c.mm.

Regarding the coagulogram, the findings are offered somewhat tentatively, but to date the degree of consistency in these findings in normal Thoroughbreds warrants a fair degree of confidence in assuming that the blood coagulation system of the normal Thoroughbred horse in the Newmarket area operates within narrow limits so far as the characteristics which have been examined are concerned. It is not yet possible to indicate with confidence what degree of departures from the normal ranges can be regarded as of clinical or pathological significance.

CONCLUSIONS

The normal range of the haemogram and coagulogram of Thoroughbred horses in the Newmarket area is defined and discussed. The results are not widely divergent from the figures

quoted for the haemograms of horses in Kentucky. The coagulograms were performed on only a small sample of Thoroughbreds but fall within quite narrow limits.

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THE PATHOGENESIS OF SHEEP POX IN THE SKIN OF SHEEP

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INTRODUCTION

Of the pox diseases which afflict domestic mammals, sheep pox is the one which causes naturally the highest mortality and a clinicopathological picture most resembling that of such severe generalised infections as variola, ectromelia and myxomatosis.

Many similarities have also recently been noted between one of the viruses causing lumpy-skin disease of cattle and other pox-group agents, especially sheep pox (Alexander, Plowright and Haig, 1957; Plowright and Witcomb, 1959). Serologically these two viruses are closely related (Plowright and Ferris—unpublished observations). The bovine disease has a widespread distribution in Africa and can assume a considerable economic importance; pathologically it shows many of the features of a generalised pox infection, including skin, mucosal and visceral lesions in which cytoplasmic inclusions can be detected (Thomas and Maré, 1945; Burdin, 1959; Burdin and Prydie, 1959).

The present limited study of the pathogenesis of sheep pox lesions, following intradermal inoculation of sheep, was undertaken with a view to relating virus growth to the cellular changes and incidentally to determine the most suitable time for the collection of specimens for histological diagnosis or virus isolation. This would probably serve as a model for future investigations of a similar character with bovine lumpy-skin disease. At the same time advantage was taken of the opportunity to study haematological reactions in sheep inoculated with sheep pox virus.

MATERIALS AND METHODS

Virus. The strain used was that described by Plowright and Ferris (1958) after 7 passages in sheep testis monolayers. The inoculum was in the form of an homogenised whole culture in LA:YE maintenance medium (Franklin, Rubin and Davis, 1957) with 5 per cent normal ox serum. It was shown in the reference quoted that the virus was not attenuated for sheep after 10 passages in sheep testis cells; it could be accurately titrated in tube cultures of the same cell type, culture titres being, in general, about 1 $\log_{10}$ unit lower than those determined by intradermal inoculation of sheep.

Experimental animals and procedure. The animals used were 8 male sheep about 4 to 6 months old; 4 of them were of the indigenous, African, fat-tailed type which has hair instead of wool, the others being high-grade Merinos. They were divided into 2 groups, each consisting of 2 African and 2 Merino animals. All were closely clipped on the right

flank over an area of about 10″ x 6″, the group for biopsies (B) being also cleanly shaved and dusted with sulphonamide powder to control minor bacterial infections. After six days, to allow the subsidence of traumatically-induced changes, one group (A) was used for observations on the pathogenicity and for titration of the inoculum in the manner described previously (Plowright and Ferris, 1958). The second group was further subdivided so that 2 sheep received undiluted inoculum (0.2 ml.) at 8 sites intradermally (group B1), and the other 2 animals a 10^{-1} dilution at 8 and 6 sites respectively (group B2). Each sheep further received 0.2 ml. of uninfected maintenance medium at 3 to 4 sites which were used as uninfected controls (Fig. 1). In group B the inoculation sites were separated by about 2 to $2\frac{1}{2}$ inches.

FIG. 1

DISTRIBUTION OF BIOPSY SITES IN SHEEP No. 32

Superior

V Day 17	C Not excised	V Day 23
V Day 11	C Day 14	V Day 4
V Day 2	C Day 2	V Not excised
V Day 7	C Day 1	V Day 1

Posterior — Anterior

Inferior

V=undiluted virus inoculum, 0.2 ml. intradermally day 0.

C=control, uninfected inoculum, 0.2 ml.

Figures refer to time of biopsy, in days post-inoculation.

The sites were clearly demarcated by grease-pencil and an elliptical area from those in group B was excised by scalpel at intervals as shown in Figs. 1 and 2. After excision the wounds were dusted with sterile sulphonamide powder and closed by sutures. Care was taken, so far as possible, to excise in the early stages the lowermost sites, to which oedema fluid would later gravitate from others higher up the flank. Details for sheep No. 32 are shown in Fig. 1. Daily clinical examinations were made to note the gross character of the reactions and to determine if generalised lesions appeared in other situations, particularly the axilla, groin, tail, scrotum and head region. All sheep were kept on daily morning temperature record.

Treatment of biopsies. Each biopsy sample was bisected immediately, half being fixed in Bouin's fluid, the other half being stored frozen at −25°C. until required for titration $4\frac{1}{2}$ to 5 months later. Fixed specimens were embedded in paraffin, sectioned at about 5μ and stained by haematoxylin and eosin.

Frozen material was weighed and ground with sand, in mortars

previously cooled to −25°C. Suspensions of 3 to 10 per cent w/v, prepared by further grinding in phosphate buffered saline (PBS : Dulbecco and Vogt, 1954), were centrifuged at 2,500 r.p.m. for 10 minutes to remove coarser particles and the supernatant was further diluted in 10-fold steps. Titrations were carried out in tubes of sheep testis cells, 5 tubes being inoculated with 0.2 ml. each of at least 4 dilutions. The maintenance medium employed was 2 per cent normal unheated horse serum in LA:YE medium (Franklin *et al.*, 1957) containing 100 units each of penicillin sodium salt and streptomycin sulphate, plus 100 units of Mycostatin*. Final readings were made on the 14th day, titres being expressed as 50 per cent cytopathogenic endpoints per gramme of tissue, calculated by the method of Thompson (1947).

Haematology. Blood samples were taken every morning from the jugular vein into oxalate mixture (Wintrobe, 1951). Determinations were made immediately on erythrocyte and total leucocyte counts together with packed cell volume. Differential leucocyte counts were made on smears stained by May-Grünwald-Giemsa, 300 cells being counted for each sample. The results were expressed as absolute numbers per cu. mm., no attempt being made to subdivide monocytes from other non-granular cells. Neutrophils were divided into segmented and juvenile forms.

* E. R. Squibb & Sons, New York.

RESULTS

General observations on the 8 sheep inoculated are summarised in Table 1. No sheep became severely sick and it can be seen that only 3 out of 8, all of indigenous African type, showed any evidence of generalisation of skin lesions. The appearance of the latter coincided with or shortly succeeded the first significant rise of temperature. They reached only a few mm. in diameter, sometimes became pustular but were in every case reduced to areas of thin, exfoliating scab by the 11th to 16th days post-inoculation.

In Group A the maximum time to the appearance of distinct local reactions was 7 days; doses in the region of 10 to 100 or less sheep ID_{50} caused visible "pock" formation after 5 to 7 days. In group B some oedematous thickening and erythema of the skin sites commenced on the 3rd day post-inoculation in all four sheep. This slowly increased in severity and extent, reaching about 3 to 5 cm. in diameter and 1 cm. in thickness by the 6th to 7th days. Towards the end of this time there was a tendency for the centre of the lesions to become slightly depressed and their colour became purplish. On the 10th day there was some exudation of clear, serum-like fluid on the free surface and this coagulated to form thin yellowish scabs; after 2 weeks the skin overlying the centre of the reaction zones had become obviously necrotic and usually formed a hard, dark scab which was delimited from the surrounding tissue by a sharp line of demarcation. The scabs could be removed manually on the 23rd day but left an underlying area of bleeding granulation tissue, together with some yellowish pus. African sheep differed

TABLE 1

Group	*Sheep No.*	*Breed*	*Days of temperature reaction**	*Peak temperature (°F.)*	*Titration result†*	*Generalisation §*	*Remarks*
A	13	African	5–8	104.4	5.1	+ (8)	Group A sheep were used
,,	14	,,	6–8	103.4	4.9	+ (8)	for titration of inoculum.
,,	30	Merino	4–9	105.2	4.9	—	Mean titre $10^{4.8}$ ID_{50} per
,,	33	,,	6–9	104.4	4.5	—	0.2 ml.
B1	11	African	6	103.6	—	—	Inoculated 8 sites each $10^{4.8}$ ID_{50}.
,,	27	,,	7	105.4	—	+ (7)	Ditto. each $10^{3.8}$ ID_{50}.
B2	32	Merino	5–10	105.2	—	—	Ditto. each $10^{4.8}$ ID_{50}.
,,	34	,,	5–7	104.8	—	—	Inoculated 6 sites each $10^{3.8}$ ID^{00}.

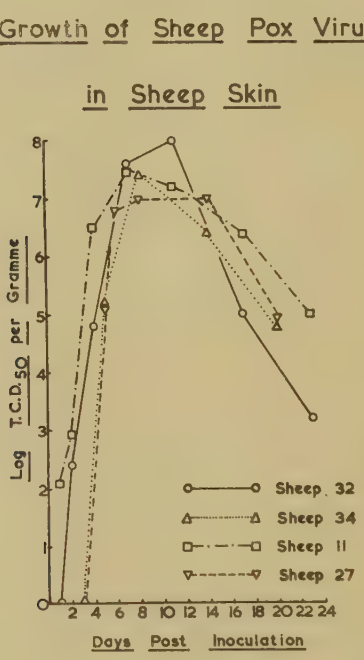
* — days post-inoculation (inclusive)

† — Log TCD50 per p.2 ml.

§ — figures in brackets refer to day of first appearance.

from the Merinos in that the scab was thinner, dry and parchment-like. Epithelial regeneration under the scabs was slow and not complete until the end of the 5th to 6th weeks. At many sites a star-shaped cicatrix was left in which there were no hair or wool follicles.

Fig. 1



Growth of Virus in Sheep Skin

The results of titrations of virus in biopsy samples are shown in Fig. 2. Virus was detected after 24 hours in one sheep which received $10^{4.8}$ ID_{50} per inoculation site (No. 11). No virus could be demonstrated after 3 days in the two sheep (Group B2, Nos. 34 and 27) given $10^{3.8}$ ID_{50} per site, i.e. there was a complete eclipse phase. A phase of logarithmic increase had commenced by the 2nd day in Group B1 and by the 5th day in Group B2. Peak titres of 10^7 TCD_{50} or more per gramme of tissue were attained on day 7 in the first, and by day 8 in the second subgroup. There was little or no fall in the quantity of virus demonstrated between the 7th and the 11th to 14th days but subsequently there was a gradual decline to the 20th or 23rd days; even then, however, the dried blackened scabs in 3 out of 4 sheep contained about 10^5 TCD_{50} per gramme. It is probable that this phase of decline is due largely to inactivation by heat and bacterial putrefaction of virus trapped in necrotic tissue; there may also be some inactivation

by neutralising antibody which diffuses throughout the tissue with the oedema fluid and serum exudates.

It was of interest that in the titration of the two 23-day samples from sheep Nos. 32 and 11 there appeared to be a finite retardation or complete suppression of the cytopathogenic effect in tubes inoculated with undiluted tissue extracts. Again there would appear to be two possible explanations for this phenomenon, either the presence of antibody derived from fluid exudates which had become dried up in the scab or the presence of viral antigens capable of interfering with viable virus but not cytopathogenic.

Histological Changes in Sheep Skin

For convenience these were best divided into those affecting mesodermal tissues of the subepithelial (papillary) layer, dermis and subcutis and secondly those involving the epithelia of the surface, hair or wool follicles and associated glands. The gross histological changes are illustrated in Figs. 3 to 6 (incl.) for a haired sheep, No. 11; Figs. 7 to 10 (incl.) show the sequence of events in a merino animal, No. 34, with special reference to the epithelia.

At sites inoculated with uninfected maintenance fluid there was a mild neutrophil leucocyte and histiocytic infiltration of the papillary layer and dermis which was present at 24 hours post-inoculation and persisted without appreciable change until the 5th day, after which no further observations were made. The inflammatory cells were found predominantly in the neighbourhood of small blood vessels; it is possible that their presence was due rather to the trauma associated with shaving than to the intradermal inoculation. The surface epithelium consisted of about 3 to 4 layers of cells, the most superficial of which were squamous and cornified (Figs. 3 and 7). The granular layer was usually only one or two cells thick and discontinuous.

The intradermal inoculation of sheep pox virus was associated with similar early changes in biopsy material but between 48 and 72 hours an oedematous infiltration began to appear in the subepithelial connective tissue, also involving the collagen bundles of the dermis (Fig. 4). At about the same time large, histiocyte-type cells, with rounded or oval nuclei and cytoplasm of increased basophilia, were seen scattered between collagen bundles and around small vessels. In the following 3 to 4 days these increased markedly in numbers and many showed distinct, rounded clearings in the nucleus with peripheral displacement of the chromatin threads and nucleoli. The nucleoli were often enlarged (Fig. 11). When this process of "vacuolation" commenced at several foci, nuclei of lobulated appearance developed. Their cytoplasmic boundaries were ill-defined but there were often long, irregular processes giving the cell a somewhat stellate appearance (Fig. 14). These elements corresponded to those described by Borrel (1903a) as "cellules claveleuses", with the exception that in many of them

it was very difficult or impossible to find cytoplasmic inclusions. In sections heavily stained with haematoxylin, granular cytoplasmic masses could sometimes be distinguished adjacent to the nucleus, such cells often showing a rarefaction of the peripheral cytoplasm (Fig. 12). The appearance of "sheep pox cells" on the 3rd to 5th days was accompanied by increasing distension of the tissue spaces by oedema fluid and by degeneration of many of the polymorphs, which fragmented forming coarse, granular debris.

The subcutaneous connective tissue and the underlying muscle showed a similar oedematous and cellular infiltration by the 6th to 7th days, the reaction being most intense around the blood vessels (Fig. 14). At this time, too, in the African sheep and a little later in the merinos (8 days or more) blood vessels in the dermis, particularly the venules, showed thrombosis accompanied by extensive degenerative changes, especially karyorrhexis, in the tissues of their walls, this process extending into the surrounding connective tissues (Fig. 13). It was not possible to find specific evidence of virus infection in the vascular endothelium or in the muscularis of these vessels, but the thrombosis almost certainly accounted for the rapid coagulation necrosis of the superficial epithelium and hair or wool follicles which, as will be noted later, began at about the same time. Between the 11th and 14th days all tissues superficial to the subcutis were necrotic, only a very few intact "sheep-pox cells" remaining in the interstices of the oedematous connective tissues. This mass became heavily infiltrated by masses of bacteria, easily visible in preparations stained by haematoxylin and eosin.

The first change in the epithelium was seen as a hyperplasia commencing on day 4 in group B1 and day 5 in Group B2. Instead of the normal 3 to 4 layers of cells there were 8 or 9, not only in the surface epithelium but also in that of the superficial parts of the follicles, down to the level of the sebaceous glands (Figs. 3 and 4, 7 and 8). Some scattered cells, at various levels, showed large clear cytoplasmic vacuoles, which displaced the nucleus laterally, but this degeneration did not have any specific features of virus-induced change. The granular layer in some instances became broader and continuous. On the 6th to 7th days in the African sheep and on the 8th day or later in the merinos, this hypertrophied epithelium had undergone coagulation necrosis with further swelling and vacuolation probably due to the imbibition of fluid (Figs. 5 and 9). The necrotic layers showed a tendency to lift and detach easily (Fig. 5). By the 11th to 14th days coagulation necrosis of the epithelia had extended to involve the roots of the hair or wool follicles, whilst still later shrinkage and condensation of the debris occurred, presumably caused by drying out.

Although at times groups of epithelial cells were seen in relation to the sebaceous glands or the deeper parts of the follicles, in which there was central clearing and a peripheral condensation of nuclear

chromatin, it was never possible to detect cytoplasmic inclusions with certainty. The sudden extensive nature of the necrosis of the epithelium and its time of onset left no doubt that it was due to primary thrombosis of the dermal vessels and not to direct action of the virus. A notable feature, clearly seen in the two series of low-power photomicrographs, was the rapid compression and collapse of the acini of the sudoriferous glands; this appeared to be brought about largely by the pressure of interstitial exudates and hyperplastic epithelium on the glandular acini.

Healing of the lesions began between the 11th to 14th days with the appearance of dense cellular granulation tissue at the boundary of the subcutis and subcutaneous muscle, the outer edge of this reaction line showing heavy bacterial proliferation and much karyorrhectic debris. At the 20th to 23rd days there was still no sign of epithelial regeneration but, as noted clinically, the dried scabs could be detached with relative ease to leave exposed a vigorous granulating base.

Haematological Reactions in Infected Sheep

A preliminary examination of the haematological data showed that there was no essential difference between the leucocytic reactions of sheep subjected to repeated biopsy and those used for titrations. This was not surprising in view of the necrosis and heavy bacterial invasion noted histologically in undisturbed lesions after the stage of vascular thrombosis—stimuli which are presumably constantly involved in natural infections and of comparable or greater severity to that involved in excision of infected sites. There was, however, a marked difference in the normal relative counts of African and

Fig. 15

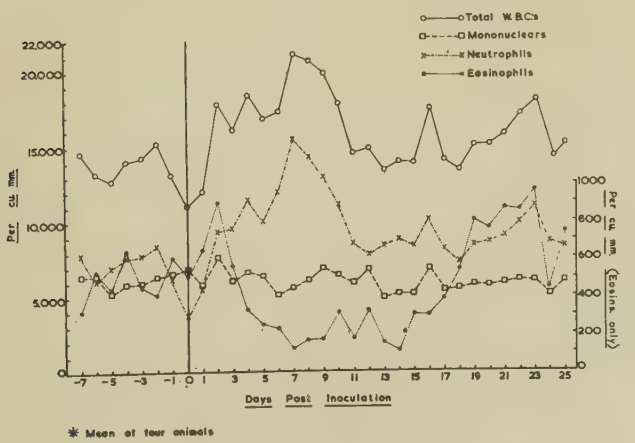
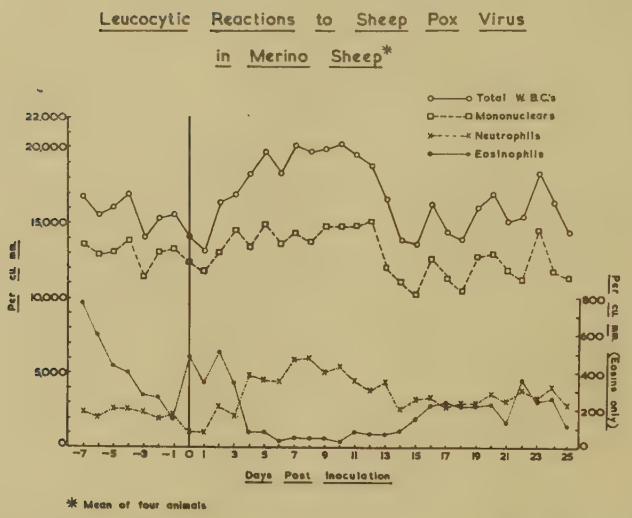


Fig. 16



Merino sheep which precluded the pooling of the figures for these two breeds. The mean total counts were approximately the same, but in Merino sheep mononuclears accounted regularly for about 80 per cent of them, whilst in African sheep they were only about 50 per cent; similar haematological observations have been made in other experiments with sheep at this laboratory. This pre-inoculation difference was associated with minor variations in the response to sheep pox infection. The results for these two breeds are, therefore, shown separately in Figs. 15 and 16.

In both types of animal there was a total leucocytosis, beginning on the 2nd day in African sheep but deferred to the 3rd and 4th day in the Merinos, and reaching a mean peak of about 20,000 per cu.mm. on the 7th day post-inoculation. Normal levels were regained on the 11th day in the former and by the 14th day in the latter. In indigenous sheep the lymphocytes remained constant throughout the period of observation and the increased total count was due entirely to a rise in neutrophil polymorphs; in the Merinos both polymorphs and lymphocytes, especially the former, showed increases of lesser degree. There was no significant "shift to the left" in either breed. Eosinophils showed a marked decrease beginning on the 4th day, with lower levels in the Merinos, which often showed a complete eosinopenia. Recovery to normal values began on the 15th to 17th days post-inoculation.

It appeared, therefore, in these infections that there was a mild reaction characterised by a leucocytosis, involving predominantly the neutrophils and beginning at about the same time as the local reactions. The peak of leucocytosis corresponded with the time of maximal development of the virus and of the local lesions.

In African sheep there were indications of a secondary rise at the time of fever and generalisation on the 6th to 9th days. Eosinopenia was a constant feature and shortly preceded the onset of fever. Recovery of the normal leucocyte picture was delayed until the time that the local lesions were showing signs of delimitation by granulation tissue and sequestration.

No significant changes in the erythrocytes were noted in the course of the experiment.

DISCUSSION

Extracts from the tissues of sheep inoculated subcutaneously with sheep-pox virus have been used for their immunisation at least since the beginning of the century, when Borrel (1903b) described the methods he had employed. He harvested lesion material at the 8th day after subcutaneous inoculation, at the time of maximal development of the "pustule". Quantitative serial studies on the proliferation of sheep-pox virus in sheep skin have not to our knowledge been reported up to the present time, presumably because of the difficulty of performing large numbers of titrations in sheep. The work reported here shows that tissue culture methods can be readily applied to the investigation of this and other similar problems since virus of the 7th culture generation was still pathogenic for sheep and did not lose its cytopathogenicity on proliferation in this species in vivo.

It is of interest to compare the results of intradermal inoculation of sheep-pox and those of Fenner (1948) after the inoculation of about 500 ID_{50} ectromelia virus into the foot-pad of mice. He noted no clinical reaction until the local concentration of virus reached about 10^7 ID_{50} per gramme on the 7th day. In sheep the local lesion was detected at about the time that virus proliferation became demonstrable and reached its maximum development at the peak of virus growth (7 to 8 days). In mice high titres were maintained until the 14th day, when antibody reached its peak and a decline of virus began. These time relationships are strikingly similar to those demonstrated for sheep-pox, where the skin virus titre remained on a plateau up to about the 14th day and then declined at approximately the same rate as it had risen. We have no detailed information on the time of development of antibody in sheep-pox, but Plowright and Ferris (1958) showed that the virus-neutralising capacity of the sera of infected sheep was developed by the 14th day and did not change materially from that time up to the 28th day. If the temperature reaction can be accepted as a sign of viraemia then the latter usually occurred in sheep 5 to 6 days after inoculation at a time when the virus had reached a local titre of 10^5 TCD_{50} per gramme or higher.

With this strain of virus the role of epithelial structures in the development of the skin lesions was a minor and secondary one, there being little or no evidence of a primary effect of the virus

on them. Although Borrel (1903a) noted proliferative and degenerative changes in the epithelium of the skin and its glands and also described in them nuclear changes similar to those in the "cellules claveleuses" together with typical cytoplasmic inclusions, he also regarded the mesodermal lesions as primary. He employed the subcutaneous route for infection but our results after intradermal inoculation pointed to a similar conclusion. The demonstration that coagulation necrosis of the skin, associated with vascular thrombosis, occurs in sheep-pox emphasises its close pathological similarity to lumpy-skin disease, where "sit-fasts" are characteristic of many cases (Henning, 1956; Burdin, 1959).

Specific cytological effects produced by sheep-pox virus in both the nuclei and cytoplasm were clearly depicted by Borrel (1903a and 1935) in lesions in sheep tissues. Similar changes have recently been described by several authors in cultured cells infected not only with sheep-pox (Aygün, 1956; Cilli and Baldelli, 1958; Plowright and Ferris, 1958) but also with a virus derived from lumpy-skin disease (L.S.D.) of cattle (Alexander *et al.*, 1957; Plowright and Witcomb, 1959) and infectious myxoma (Chaproniere 1956).

Thomas and Maré (1945) described cytoplasmic inclusions in the lesions of lumpy-skin disease of cattle; one of us (Plowright, 1957—unpublished observations) confirmed the presence of these and noted nuclear clearings in infected cattle tissues examined at Onderstepoort, whilst Burdin (1959) has recently found identical changes in material from Kenya cases. Rivers (1930) described swollen and "vacuolated" nuclei in skin epithelial cells of rabbits infected with myxomatosis but still earlier Hobbs (1928) depicted similar cells and appended a footnote "Nuclei of affected cells often vacuolated". Hurst (1937) observed dense nuclear membranes, chromatin fragmentation and oxyphilic granules within the nuclei of occasional myxoma cells, and also in epithelial, endothelial or mesothelial elements; very rarely he found inclusions simulating those of Borna disease. Epstein, Reissig and De Robertis (1952) noted that typical myxomatous cells examined by electron micrography showed a thickening of the nuclear membrane, apparently due to the deposition on it of an amorphous material of high electron density. Nuclear changes similar to those in sheep-pox and L.S.D.-infected cells were seen by Blakemore and Abdussalam (1956) in swine-pox-infected tissues.

It must be concluded that all these pox-viruses have closely comparable cytological effects but we consider that the term "vacuolation" is not one to be recommended for the intranuclear changes. Some eosinophilic granular material, occasionally aggregated to form a distinct "inclusion body", can always be found within the so-called "vacuoles" of sheep-pox-infected culture-cells. The latter, of course, offer a far better opportunity for

study of the problem than pathological tissues fixed as relatively thick slices.

A discussion of the origin of the "cellules claveleuses" would appear to offer as many opportunities for controversy (see Hyde and Gardner, 1933) as that of the large stellate cells in myxomatosis. Hurst (1937), who considered the latter problem, concluded that "there is a fundamentally similar response to infection on the part of fibroblasts, adventitial elements and vascular and reticular endothelium, all of which tend to develop along similar lines". Borrel (1903a) merely stated that the "cellules claveleuses" were of mesodermal origin. We formed the impression that the majority present in the skin arose from fibrocytes lying between collagen bundles but large numbers were also found in situations suggesting an origin from undifferentiated perivascular cells; there was no evidence of vascular changes similar to those described by Hurst (1937) in myxoma.

CONCLUSIONS

An account is given of the results of inoculating sheep pox virus intradermally in African and Merino sheep. Serial skin biopsies were performed at intervals over a period of 23 days post-inoculation and each sample was titrated for virus content and examined histologically. Clinical reactions of the sheep are also described.

A complete eclipse phase was demonstrated in 3 out of 4 animals. It was succeeded by a phase of logarithmic increase, peak virus titres of over 10^7 TCD_{50} per gramme of tissue being attained on the 7th to 8th days. This high level was maintained until the 14th day after which a slow decline began. About 10^5 TCD_{50} virus per gramme still remained in the dried scabs present on the 20th to 23rd days. The probable reasons for the phase of decline are mentioned.

The main histological changes took place in the mesodermal tissues of the subepithelial layer, dermis and subcutis. There was considerable oedema and the production of large numbers of cells described by Borrel as "cellules claveleuses". These showed constant nuclear changes which are described and discussed in detail; granular cytoplasmic inclusions were sometimes observed. On the 6th to 8th days, in one case later, extensive thrombosis of dermal blood vessels occurred.

With this strain of virus and in the conditions stated, epithelial changes consisted of a primary mild hyperplasia beginning on the 4th to 5th days followed by coagulation necrosis, coinciding with and apparently caused by the thrombosis noted above. Specific signs of virus infection were not observed in epithelia.

Daily haematological examinations were carried out on 8 sheep over the period of the experiment. There was a marked and consistent difference between the normal differential leucocyte counts

of African and Merino sheep. Infected sheep of both breeds showed a mild leucocytosis affecting predominantly the neutrophil polymorphs and coinciding with the period of development of the local lesions. There was also an eosinopenia.

No significant changes in the erythrocyte picture were recorded.

ACKNOWLEDGMENTS

We are grateful to Mr. M. L. Burdin and Mr. J. Prydie for permission to quote from papers which are not yet published. Thanks are also due to Dr. R. A. Alexander, Onderstepoort for the opportunity to examine tissues from cases of lumpy-skin disease.

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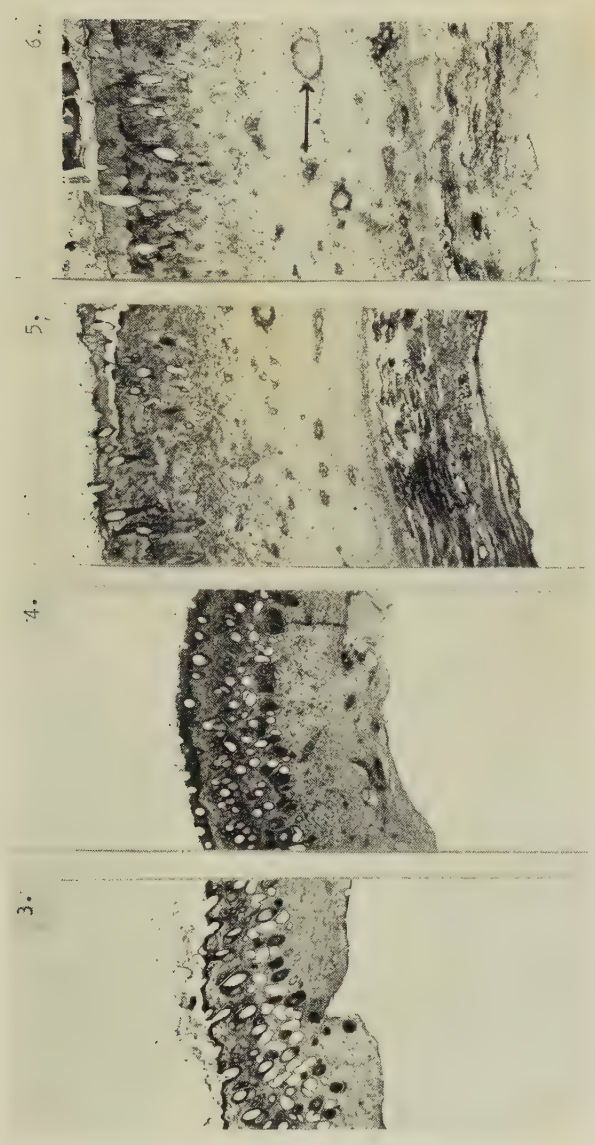

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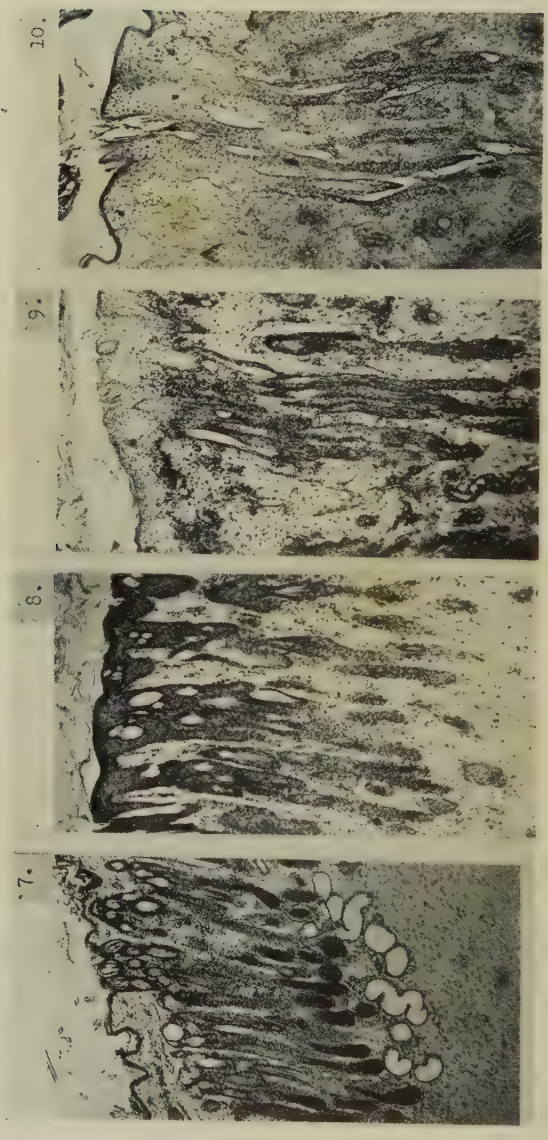
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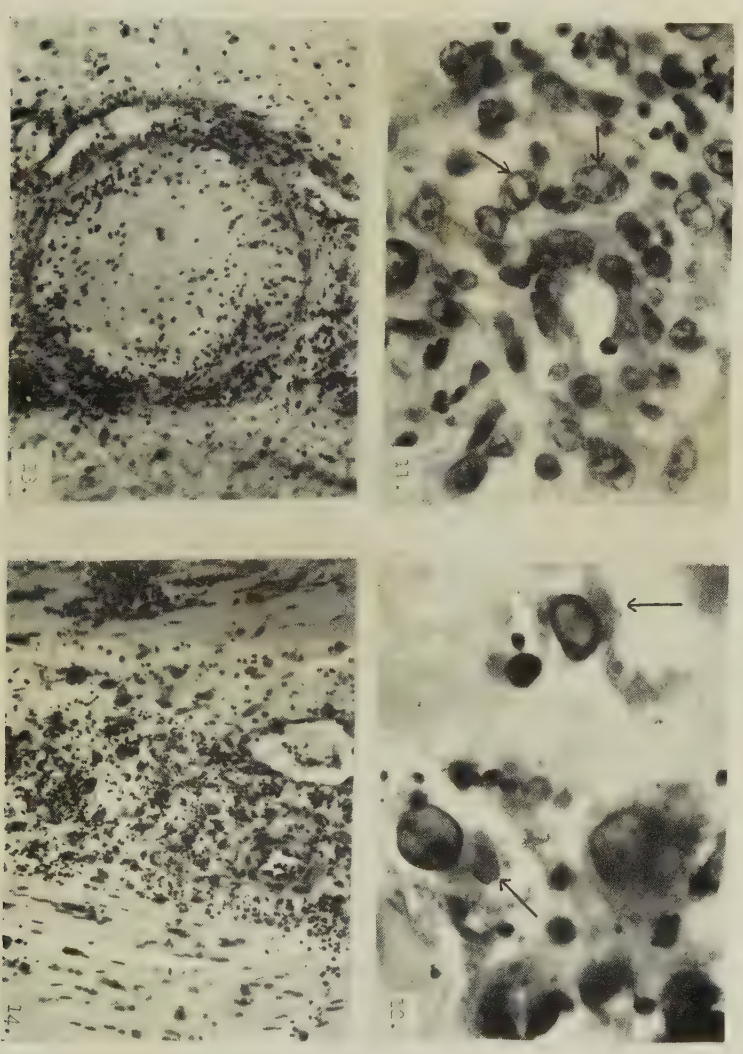

EXPLANATION OF PLATES

All figures are of tissues fixed in Bouin's fluid and stained by Ehrlich's haematoxylin and eosin.

Fig. 3. Section of skin biopsy of African sheep (No. 11) 4 days following inoculation of uninfected maintenance fluid. (x 10).

Fig. 4. Same sheep, 4 days after inoculation of $10^{4.8}$ sheep ID_{50} of cultured sheep-pox virus. Note thickening of surface epithelium, cellular infiltrations of subepithelial tissue and dermis. (x 10).

Fig. 5. Sheep 11, day 7 post-inoculation. Necrosis and detachment of surface epithelia; gross thickening of dermis and cellular infiltration of subcutaneous muscle. (x 10).

Fig. 6. Same sheep, 11-day lesion. Great swelling and necrosis of epidermis and dermis; destruction of subcutaneous muscle. Large vessels of dermis show obvious thrombosis (arrowed). (x 10).

Fig. 7. Biopsy from control non-infected site of Merino sheep No. 34, 5 days following inoculation of culture maintenance fluid. (x 30).

Fig. 8. Same sheep, 6 days after inoculation of $10^{3.8}$ sheep ID_{50} of cultured sheep pox virus. Note hypertrophy of epithelium and virtual disappearance of sudoriferous glands. (x 30).

Fig. 9. Sheep 34, day 8 post-inoculation. Necrosis of surface epithelium and superficial part of follicles. Dense subepithelial cellular infiltrations. (x 30).

Fig. 10. Same sheep, 14-day lesion. Necrosis of wool follicles extending throughout their depth. Hydropic swelling of surface epithelium. (x 30).

Fig. 11. Same preparation as Fig. 4. Small vessel at centre with histiocytic cells (arrowed) showing early nuclear clearings and large nucleoli. Note also nuclear debris from polymorphs. (x 600).

Fig. 12. "Cellules claveleuses" showing heavy condensation of chromatin on nuclear membrane. Two also have granular cytoplasmic inclusions (arrowed). Same preparation as Fig. 5. (x 1200).

Fig. 13. Dermal vessel showing thrombosis and necrosis of cellular elements in its wall. Same preparation as Fig. 5. (x 140).

Fig. 14. Subcutaneous muscle with heavy perivascular and interstitial infiltration by stellate "sheep-pox cells" and polymorph debris. (x 140).

EXPERIMENTAL INFECTION OF LAMBS WITH *MUELLERIUS CAPILLARIS*

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INTRODUCTION

The sheep lungworm, *Muellerius capillaris,* has been recorded from many parts of the world including Europe, Australia, Asia, S. Africa, and the U.S.A. In some of these regions the incidence of the parasite is very high.

According to Mapes and Baker (1950) nearly all lambs over six months old, slaughtered in the eastern U.S.A., are infected. Investigations carried out at Weybridge suggest an equally high incidence in this country (Rose, 1955). Very little appears to be known about this lungworm despite its high incidence. There is no general agreement concerning the effect of infection on the health of the sheep. Cameron (1933) maintains that it is doubtful whether *M. capillaris* causes any symptoms as a rule, although heavy infections would assist in causing debility and chronic pneumonia. Somewhat similar opinions are expressed by both Lapage (1956) and Mönnig (1957). American workers think differently, however. Mapes (1948) states that *M. capillaris* is considered to be the cause of extensive loss to sheep and goat breeders in all parts of the world, and he quotes Professor Baker of Cornell University as saying that it is a limiting factor in the production of choice market lambs wherever it is endemic. As these various opinions do not appear to be based on experimental investigations, it is not possible to decide which is correct.

In view of the high incidence of *M. capillaris* in this country, it is clearly desirable to have more definite information about the effect of this lungworm on the health of the host. The most satisfactory way of obtaining this is by controlled experimentation. In carrying out such experiments, it is essential, for the results to have any practical significance, that the infection produced in the experimental animal should be comparable with the naturally acquired infection. As is well known, the sheep becomes infected by accidentally ingesting the molluscan intermediate host. Little else, however, is generally known about this lungworm, so that a brief account of some findings relating to the epidemiology of natural infections seems necessary if the methods of experimentation are to be understood.

A number of workers have demonstrated that the percentage infection of the population of molluscs in a given area is not usually high, and that an infected mollusc generally harbours only a small number of lungworm larvae (Rose, 1957). Consequently, there is little chance of a sheep picking up a large number of infective larvae in a short time, as may occur with most other sheep nematodes, the infective larvae of which are free-living on the herbage. This means that a heavy infection will be built up over a relatively long period of time. Post-mortem examina-

tion of sheep of different ages has given the impression that this does in fact happen.

In naturally infected sheep slaughtered at Watford abattoir there were considerable variations in the surface area of the lungs covered by the nodules which result from infection with *M. capillaris*. This was due to differences both in size and number of the nodules present (Rose, 1955). In order to record this variation, the severity of infection was graded into 'light', 'medium' and 'heavy'. 'Light' infections were classified as those having not more than 50 small superficial nodules. 'Medium' infections were those in which small superficial nodules were more numerous: most of the lungs so graded had between 200 and 600. 'Heavy' infections included those in which large superficial nodules, between 1 and 5 cm. in diameter were present on the lungs, and also those in which there were over 1,000 small nodules. Of the Watford sheep examined, 1733 had 'light' infections, 2038 had 'medium' infections and 181 had 'heavy' infections. The amount of lung tissue rendered inactive in the lungs of those animals with 'light' infections was so small that it seemed unlikely that the health of the host would be affected. With the 'medium' and 'heavy' infections, however, a considerable amount of lung tissue was inactivated and it seemed likely that the health of the host would be affected.

Having regard to these various observations it seems that in general it is improbable that lambs will often acquire 'heavy' infections of *M. capillaris*. 'Light' infections are likely to be common but unlikely to prove harmful. 'Medium' infections, however, may be both harmful and common. It would seem, therefore, that a study of 'medium' infections in lambs is needed. The present paper is an account of such a study.

METHODS

In the first experiment three groups of lambs, approximately 4 months old, reared indoors and free of infection except for very light infections of *Strongyloides papillosus* and coccidia, were infected with *M. capillaris* as follows. Group 1 consisted of lambs numbers 1 to 4. Each was given between 60 and 70 infective larvae daily on five consecutive days per week, for a period of 44 weeks, making a total of between 13,200 and 15,400 larvae. Group 2 consisted of lambs numbers 5 to 7. Each was given between 60 and 70 larvae once per week for 44 weeks, making a total of between 2,640 and 3,080 larvae. Group 3 consisted of lambs numbers 8 to 12. Each was given a single dose of approximately 3,000 larvae at the outset. Two other lambs, numbers 13 and 14, were uninfected.

All of the lambs were kept together indoors as a single group under conditions which precluded accidental infection. Frequent observations were made for any obvious clinical symptoms such as coughing or distressed breathing. At intervals of 14 days the lambs were weighed. after being deprived of food and water overnight. At weekly intervals a 5 g. sample of faeces was taken from the rectum of each lamb and any larvae present were separated and counted. At the end of 44 weeks, the lambs, except for number 11, which was still passing first stage larvae, were autopsied and the lungs examined. The superficial nodules

were counted and a representative 20 per cent sample examined microscopically. Lamb 11 was kept alive to see for how long it would continue to pass first stage larvae.

The second experiment was prompted by the results of the first. It was designed to compare the weight gains of infected and uninfected lambs.

Four pairs of twin lambs, comprising two pairs of ewes and two pairs of wethers, were divided into two groups A and B, with one twin to each group. In each pair, one lamb weighed more than its twin, but they were divided to give, as far as possible, two balanced groups. Each lamb in Group A was given approximately 100 infective larvae daily on five consecutive days per week for 36 weeks, making a total of approximately 18,000 larvae. The group B lambs were uninfected. The lambs were reared and kept under conditions similar to those operating in the first experiment. They were weighed and re-weighed immediately before the first and after the last dose of larvae. The infected lambs were then autopsied and the lungs examined as before.

Details of all the techniques used in these experiments have already been published (Rose, 1957, 1958) and are not repeated in this paper.

RESULTS

Clinical Symptoms

At no time were any obvious clinical symptoms such as coughing or distressed breathing observed, even after the lambs had run around their pen. Both infected and uninfected lambs were in good condition, and it was not possible to tell from external appearance which was which.

Weight Gains

The weight gains of the lambs in the first experiment are recorded in Table 1. As this experiment was not designed specifically to study weight gains, no definite conclusions can be drawn from the results. As the best weight gains were made by the uninfected lambs, however, further experiments were considered to be worth-

TABLE 1

WEIGHT GAINS OF LAMBS

Experiment 1

	Infected											*Uninfected*	
Group number	*1*					*2*		*3*				*4*	
Lamb number	1	2	3	4	5	6	7	8	9	10	11	12	13
Initial weight lb.	46	49	62	72	41	46	56	58	47	46	61½	59	61
Final weight	93½	82	122	129	83	102	90½	112	90	87	95	122	125
Weight gain	47½	33	60	57	42	56	34½	54	43	41	33½	63	64

TABLE 2

WEIGHT GAINS OF LAMBS

Experiment 2

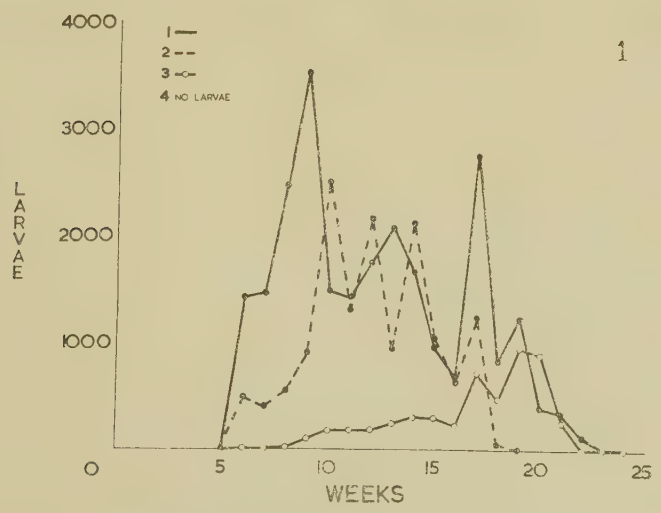
	Group A infected				*Group B uninfected*			
	Wether		*Ewe*		*Wether*		*Ewe*	
Lamb number ..	1	2	3	4	1	2	3	4
Initial weight, lb. ..	73½	74	57½	70	68	76½	54	75
Final weight ..	134	136	87½	102	133	146½	96	120
Weight gain	60½	62	30	32	65	70	42	45
Average weight gain	46.1 lb.				55.5 lb.			

while. The weight gains made during the second experiment are recorded in Table 2. The wethers gained considerably more than the ewe lambs, but each uninfected lamb gained more than its infected twin. The average weight gain of the uninfected lambs was 9.4 lb. more than that of the infected lambs.

Output of First Stage Larvae

The numbers of first stage larvae recovered from the faecal samples taken during the first experiment are depicted in Figs. 1 to 4. Larvae were recovered from all of the infected lambs

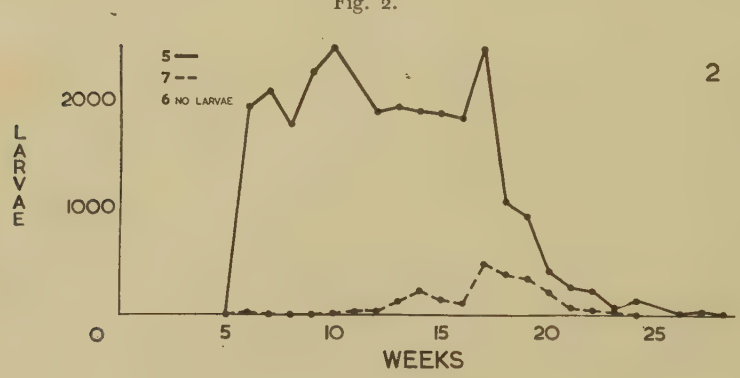
Fig. 1.



Larvae per 5g. of faeces taken at weekly intervals from lambs 1 to 4.

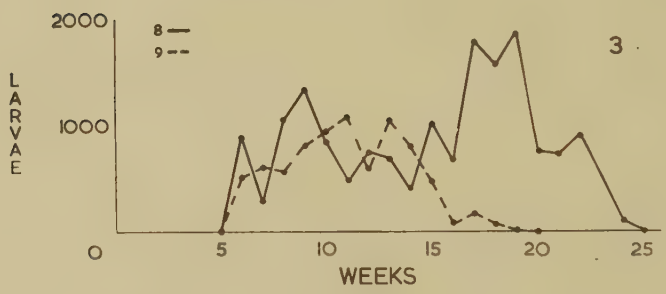
Figs. 1 to 4. Faeces were taken until the lambs were autopsied. The output of larvae ceased as indicated in Figs. 1 to 4. The subsequent zero points are not recorded. Lamb 11 passed larvae continuously for over two years.

Fig. 2.



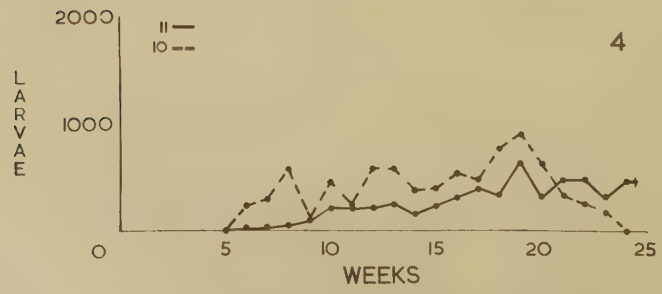
Larvae per 5g. of faeces taken at weekly intervals from lambs 5 to 7.

Fig. 3.



Larvae per 5g. of faeces taken at weekly intervals from lambs 8 to 9.

Fig. 4.



Larvae per 5g. of faeces taken at weekly intervals from lambs 10 to 11.

except numbers 4 and 6. They were first observed six weeks after the initial infection and continued to be passed for between 14 and 22 weeks, with one exception, (No. 11). This lamb was not autopsied. It has passed larvae continuously for two years and is still doing so at the time of writing.

Post-mortem Examination

Nodules resulting from infection with *M. capillaris* were present in the lungs of all the infected lambs. The numbers of superficial nodules are recorded in Table 3. Some nodules were present within the lungs. No attempt was made to count these accurately in view of the difficulty in examining all of the internal lung tissue; they were, however, much less numerous than the superficial nodules, which varied both in size and structure. In the group 3 lambs, which received only a single dose of larvae, the nodules were between 3 and 5 mm in diameter. The majority were calcified and contained either living or disintegrating adult worms. The remainder were non-calcified and contained living adults. The size range of the nodules in all of the lambs which received frequent doses of larvae varied from less than 1 mm to 5 mm in diameter, forty to fifty per cent were non-calcified, and contained worms in all stages of development ranging from immature forms only 720 μ long to fully grown adults up to 25 mm long. Living adults were present in the two lambs, 4 and 6, from which no first stage larvae were ever recovered. Most of the calcified lesions contained disintegrating worms, but a few contained living adults.

TABLE 3

A COMPARISON OF THE NUMBERS OF INFECTIVE LARVAE ADMINISTERED AND THE NUMBERS OF SUPERFICIAL NODULES IN THE LUNGS OF INFECTED LAMBS

	Experiment 1											*Experiment 2*			
Group	*1*				*2*			*3*				*A*			
Number of infective larvae administered.	13,200—15,400				2,640—3,080			3,000				18,000			
Lamb Number ..	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4
Number of superficial nodules ..	788	928	615	287	647	403	305	297	412	372	Not Killed	410	695	535	576
Approximate percentage of larvae established in the lungs. Average per group	4–5				15–17			12				3			

$$\frac{\text{Average number of superficial nodules per. gp.} \times 100}{\text{Number of infective larvae administered.}} = \text{Average percentage of larvae established in lungs (Approx.).}$$

Nearly all of the lesions, both calcified and non-calcified, contained but a single worm; only a few contained more than one.

DISCUSSION

Under the conditions of the experiments, lambs were able to tolerate 'medium' infections of *M. capillaris* without exhibiting any obvious clinical symptoms. The weight gains of infected lambs were not, however, as good as those of uninfected lambs. This observation seems to support Baker's view (Mapes, 1948) that *M. capillaris* is a limiting factor in the production of choice market lambs. Although *M. capillaris* is not likely to be the direct cause of extensive losses amongst lambs in this country, the possibility of infection with lungworms facilitating secondary infection with bacteria or viruses cannot be excluded.

The observations on the output of first stage larvae and the post-mortem examinations have a number of interesting implications. In previous observations (Rose, 1958), worms, both mature and immature, were found in nodules only. None were ever found in healthy lung tissue. From the observations made during the Watford survey (Rose, 1955), it was estimated that between 80 and 90 per cent of the nodules in the lungs were superficial in position. In the present experiments also, the majority of the nodules were superficial. The numbers of superficial nodules do, therefore, give a good indication of the numbers of worms in the lungs, although they do not correspond exactly. A comparison of these numbers (Table 3) with the numbers of first stage larvae recovered from the faecal samples, (Figs 1 to 4), shows that the two cannot be related. The extreme instances are lambs 4 and 6 which had 287 and 403 nodules respectively, some of which contained living adult worms; yet no first stage larvae were ever recovered from the faeces of these two lambs. The absence of larvae from the faeces did not, therefore, signify freedom from infection. Similarly, the numbers of larvae recovered from the other infected lambs were not related to the numbers of worms in the lungs. The explanation of this apparently anomalous result lies in the site and mode of development of *M. capillaris* whereby many worms in the lungs become isolated each in its own nodule (Rose, 1958). As a result many female worms fail to produce fertile eggs.

As suggested by Rose (1958) the importance of these observations lies in the interpretation of the results of faecal examinations. Not only are faecal examinations used as an aid to diagnosis, but also as a means of assessing the degree of infection and as an index of the efficacy of new anthelmintic drugs. At all times, caution must be exercised in interpreting the results of faecal examinations. This is particularly important when dealing with *M. capillaris*. The length of time during which larvae were excreted is such as to suggest that infection can be carried over from one season to

the next by carrier animals. This has, in fact, been observed to occur in a naturally infected flock, in which both ewes and yearlings acted as carriers.

It has been observed already that the numbers of superficial nodules in the lungs correspond closely to the numbers of worms in the lungs. It follows, therefore, that they correspond similarly to the numbers of infective larvae which reached and became established in the lungs. A comparison of the numbers of superficial nodules with the numbers of infective larvae administered (Table 3) shows that many larvae failed to become established in the lungs. In the case of the Group 3 lambs, which received only a single dose of infective larvae, it is probable that many were merely unsuccessful in following the migratory route to the lungs. In those lambs which received many doses of larvae (Groups 1 and 4) an appreciably smaller percentage reached the lungs than in the other lambs (Table 3). This would seem to indicate the intervention of some resistance mechanism limiting the numbers of larvae which reached and became established in the lungs of these lambs.

A build up of infection did take place, however. This is best illustrated by the lambs of group 2, (Table 3) which received 60 to 70 infective larvae, once per week for 44 weeks. Even if it were assumed that all of the larvae given at any one time reached the lungs, (which is improbable in view of the evidence from the group 3 lambs), it would have taken several weeks for larvae to have reached the lungs in numbers sufficient to account for the nodules present. For example, lamb 5 had 647 superficial nodules. It would have taken a minimum of ten weeks for this number of larvae to have reached the lungs. The presence of worms in all stages of development, including very immature forms only 720 μ long, further suggests that larvae had continued to reach the lungs throughout the period of infection, although in this instance a similar effect would have been noted if inhibition of development had occurred.

These observations suggest a resistance mechanism which limits but does not completely prevent larvae from reaching the lungs. It seems that a build up of infection can take place and that heavy infections are gradually developed over a period of time.

CONCLUSIONS

Lambs were infected with *M. capillaris*. No obvious clinical symptoms resulted, but uninfected lambs made better weight gains than infected lambs.

Faecal samples, taken at weekly intervals, were examined for first stage larvae. The numbers recovered were not related to the numbers of worms observed in the lungs at autopsy.

Adult worms only were present in lambs which received a single

infection. Worms in all stages of development were present in lambs which were given infective larvae at frequent intervals.

The significance of these findings is discussed.

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EFFECT OF CAPRINIZED RINDERPEST VIRUS IN RABBITS

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INTRODUCTION

Strains of rinderpest virus which differ antigenically have never been recognized and live viruses attenuated either in the goat or the rabbit have been used successfully to control the disease in cattle. Moreover, the attenuated strains injected into cattle will interfere with virulent virus before active immunity is established (Pfaff, 1938; Brotherston, 1951). Successful interference suggests that the attenuated strains utilize the same cellular metabolic pathways as virulent virus. In other hosts, however, strains differ in their behaviour and the present paper records the completely negative response of rabbits to the caprinized strain of rinderpest virus.

MATERIALS AND METHODS

Viruses. Caprinized and lapinized strains of rinderpest virus were used. The caprinized virus was the VK substrain and had been passaged serially through more than 600 goats. The lapinized virus was the Nakamura III strain which had been passaged 800 times in rabbits. Aliquots of each strain were stored as freeze-dried infected tissues at minus 20°C. until required.

Animals. European rabbits, *Oryctolagus cuniculus*, reared in Kenya were inoculated when four to six months old and weighing 1·5 to 2·0 kg. When given 1,000 rabbit ID_{50} or more of lapinized rinderpest virus these rabbits develop a fever, the experimental limits of which are accurately known, and pathognomonic visceral lesions (Scott and MacLeod, 1955).

The goats originated from the Eldama Ravine area of Kenya. They were small, compact, multi-coloured animals with short hair. The cattle were the progeny of East African Short-horned Zebus crossed with animals belonging to various breeds of European origin.

Susceptibility experiments. Rabbits were inoculated intravenously with 1·0 ml. of caprinized virus calculated to contain 100 to 1,000 cattle ID_{50}. The viabilities of the inocula were checked simultaneously in cattle. Some rabbits were sacrificed at varying intervals after inoculation. Some were bled for serum and others were challenged by the intravenous inoculation of 1,000 to 10,000 rabbit ID_{50} of lapinized rinderpest virus. The rabbit sera were examined for rinderpest antibodies by a serum constant-virus dilution technique, the indicator system of which was based on the response of rabbits to lapinized rinderpest virus.

Recovery experiments. Most rabbits were first inoculated with caprinized

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of lapinized rinderpest vaccine were titrated simultaneously in 20 previously uninoculated rabbits and in 20 rabbits given caprinized virus 18 and 24 hours before.
virus and 18 to 72 hours later were re-injected with lapinized virus. In others, both strains were injected simultaneously. Finally, two batches

RESULTS

Response to Caprinized Virus

Neither fevers, clinical signs nor deaths occurred in 27 rabbits inoculated intravenously with caprinized rinderpest virus. Sixteen of the rabbits were killed at periods ranging from 1 to 13 days after inoculation. None had lesions attributable to rinderpest. Eleven rabbits were challenged 5 days after inoculation and all proved susceptible to lapinized rinderpest virus. Neutralizing antibodies were not detected in rabbit sera taken 12 days after the inoculation of caprinized virus (Table 1).

TABLE 1

NEUTRALIZATION INDEX

Pooled sera of three rabbits taken 12 days after the inoculation of caprinized rinderpest virus

Dilution of lapinized virus	*Infected/Inoculated*	
	Pooled sera plus virus	*Normal rabbit serum plus virus*
10^{-3}	4/4	4/4
10^{-4}	4/4	4/4
10^{-5}	0/4	2/4
10^{-6}	0/4	0/4
ID 50	$10^{-4.5}$	$10^{-5.0}$
Neutralization index	<10	

Effect on the Virus

Bloods and suspensions of spleens, lymph glands, appendices, brains and spinal cords from 4 rabbits injected with caprinized virus failed to infect groups of 6 goats. Several goats died from intercurrent infections but all the survivors proved to be susceptible to rinderpest. This inability of the caprinized virus to survive one passage in rabbits was confirmed by repeating the experiment in cattle. These likewise proved to be susceptible when challenged with virulent rinderpest virus.

Interference Experiments

Interference with the growth of lapinized rinderpest virus by prior or simultaneous inoculation of caprinized virus did not occur. Six rabbits died from shock immediately after the injection of the lapinized virus. The remaining 17 rabbits reacted (Table 2). Moreover, the mean thermal response of the doubly inoculated rabbits did not differ significantly from the expected response to lapinized virus injected alone (Table 3). Finally, the prior exposure of rabbits to caprinized virus did not influence lapinized virus titrations (Table 4).

TABLE 2

INTERFERENCE

Failure of the prior administration of caprinized rinderpest virus to interfere with lapinized rinderpest infections in the rabbit

Interval between inoculations (hours)	*Number inoculated*	*Number reacting to lapinized virus*
Simultaneous	4	4
18	3	3
24	3	3
66	1	1
72	6	6

TABLE 3

THERMAL RESPONSE

Mean thermal response (°F) after inoculation with lapinized rinderpest virus of 17 rabbits previously given caprinized virus compared with the expected thermal response to lapinized rinderpest virus injected alone

Hours after lapinized virus inoculation	*Rabbits given both viruses*	*Rabbits given lapinized virus along*	*Significance of difference*	
			t	*P*
1	103.2 ± 0.2	103.2 ± 0.2	1.337	>0.1
16	102.4 ± 0.5	102.3 ± 0.1	0.496	>0.5
25	102.9 ± 0.2	102.8 ± 0.1	0.860	>0.3
40	105.0 ± 0.3	105.2 ± 0.2	0.977	>0.3
49	105.5 ± 0.3	105.6 ± 0.2	0.352	>0.5
64	105.1 ± 0.5	105.4 ± 0.2	1.308	>0.2

TABLE 4

TITRATIONS

Effect of the prior exposure of rabbits to caprinized rinderpest virus on the titrations of lapinized rinderpest vaccine

Batch number of lapinized vaccine	*Titre* in "normal" rabbits*	*Titre* in "caprinized" rabbits*	*Significance of difference*	
			t	*P*
A	3·9	3·7	0.963	>0.30
B	3·9	3·5	2.158	>0.05

* Titres expressed as the negative logarithm of the 50 per cent end-point dilutions.

DISCUSSION

The lack of clinical signs and post-mortem lesions, the failure to develop antibodies and the response to challenge with lapinized rinderpest virus indicated that rabbits were not infected or immunized by caprinized rinderpest virus. Moreover, the caprinized virus did not interfere with the establishment of lapinized rinderpest infections in rabbits and it was not recovered after one passage in rabbits.

Edwards (1949) maintained that the adaptation of a bovine strain of rinderpest virus to goats yielded a complex containing bovine and caprine moieties. He passaged his caprinized strain in rabbits and got essentially similar results to ours which he interpreted as indicating that one rabbit passage split the complex into its two moieties. The bovine moiety was lost but the caprine component remained and was capable of infecting goats which were nevertheless still susceptible to the original caprinized strain containing the bovine moiety. We suggest that caprinized rinderpest virus is non-pathogenic for, and unable to propagate in, rabbits.

CONCLUSION

The VK strain of caprinized rinderpest virus failed to infect or survive in rabbits.

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